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THE RAT VIRUS

F. G. NOVY, W. A. PERKINS, R. CHAMBERS AND P. H. DeKRUIF
From the Department of Bacteriology, University of Michigan, Ann Arbor, Michigan

It was customary in the laboratory to maintain spirochetes and trypanosomes in white rats by serial passage. For this purpose, blood was drawn from the tail or from the heart and injected intraperitoneally into clean rats. This procedure was repeated every 2 or 3 days.

One of the spirochetal strains was originally isolated in East Africa by Robert Koch and taken by him to Germany. This strain was received in Ann Arbor on February 28, 1907, and was maintained, as stated, by serial passage in rats. At first, there was no difficulty in doing this, but in the latter part of August, 1909, it was found that the rats would die in 2 to 4 days after inoculation, at times with very few spirochetes in their blood. The strain of spirochetes, however, was maintained with some effort until the latter part of October, when the rats were dying in 2 days, or less, with very few or no spirochetes in their blood.

It clearly was necessary to do something to save the strain. Accordingly, a white rat and a white mouse were inoculated with stored blood 6 days old. The rat died in 36 hours with very few spirochetes in its blood. The white mouse in 2 days showed 2 spirochetes per field under the microscope. It was then bled and its blood was injected into 2 white rats. These developed the typical rich infection with spirochetes and the strain was thus re-established and has been maintained ever since without any recurrence of the secondary or mixed infection.

Having thus, by mouse passage, saved the spirochete strain, the question then arose as to the nature of the contaminant.

Direct examination of the blood of infected rats or of stained preparations failed to reveal any organism other than the spirochete. The Zeiss dark-field and the ultraviolet microscope revealed nothing. Likewise, all attempts at cultivation, under aerobic and anaerobic conditions, on a variety of liquid and solid mediums proved fruitless. The blood of the infected rats was clearly free of bacteria and this fact led to the belief that a filter-passer was present. Accordingly, experiments were made which confirmed this view.

FILTRATION

Berkefeld filters.—In the first series of 16 trials, small Berkefeld filters were used. For each test about 2 cc of the blood of an infected rat were diluted (1:10) with sterile sodium citrate solution. In every case, the filtrate proved to be infective for rats, producing typical symptoms and death in 1½ to 2½ days. The amount of the filtrate injected varied from 0.25 cc to 5 cc.

Pasteur Chamberland filters.—Having

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A brief mention of the rat virus was made in two general addresses which appeared in:

1. Physician and Surgeon, 1911, **33**: 243; Trans. Clinical Society, University of Michigan, 1911, **182**.
2. Michigan Academy of Science, 1911, **31**.

In these the smallness of the virus was stressed, as shown by its passage through filters, collodion sacs and agar; by its nonsedimentation at 8000 r.p.m.; and by its M.L.D. No details were given. The apparent loss of the virus precluded a detailed publication. But its recent recovery by Russell T. Jordan et al. (pp. 124–129), justifies the present paper.

TABLE 1.—*Filtration through agar, April 20, 1911.*

Test	Air pressure in pounds	Filtrate (1 cc in 2 hr.)	Injected	Result
	Gravity			
A	2½	10 cc in 1½ hr.	Rat 1.73.20	*46 hrs.
B	5	10 cc in 1 hr.	Rat 2.73.00	*44 hrs.
C	7½	27 cc in 1 hr.	Rat 3.73.20	*43 hrs.
D	10	12 cc in 25 min.	Rat 4.73.20	*48 hrs.
		Control	Rat 5.73.20	*45 hrs.

The control rat was injected with 2½ cc of the unfiltered virus dilution.
* Indicates that animal died.

established the presence of a filterable virus by its passage through Berkefeld filters, the next step was tc confirm this by the use of Pasteur-Chamberland bougies, sterilized in a dry-heat oven at 170 C for 30 minutes. The infective rat blood was diluted with 10 to 20 parts of citrate solution and a culture of *Staphylococcus aureus*, as a test organism, was added, and the mixture was then centrifugated for 3 hours at 4000 r.p.m. The supernatant was then introduced into the filtering apparatus and suction applied.

Three tests were made with the Pasteur F filter and three with that marked B. All 6 filtrates were sterile as regards the presence of *Staph. aureus* but they infected rats, producing the usual symptoms, and death in 2¼ to 4½ days. One filtration experiment was made with *undiluted* serum and the least porous Pasteur B filter. The culturally sterile filtrate was injected into 2 rats and caused typical death in 2½ and 3½ days.

Collodion sac filtration.—Having successfully passed the virus through the densest porcelain filters, the next step was to ascertain whether or not it would pass through a collodion membrane. Collodion sacs had been used in the laboratory for some years, especially for dialysis. The method of preparing the sacs was described by C. S. Gorsline[1] and used by D. J. Levy.[2] The question of the porosity of collodion membranes had been studied by F. E. Bartell.[3]

The collodion sac was about 0.5 inches in diameter and 2.5 inches long. It was mounted on a glass tube which was provided with a rubber stopper fitting a large test tube on foot, which had a side-arm. The sac and receiver were then partly filled with water and sterilized at 105 C for 10 minutes, after which the water was removed.

The pooled blood from several infected rats was first centrifugated at 4000 r.p.m. for 10 minutes; the clear serum was then removed and diluted with 10 or 15 parts of sterile 1% salt solution. The diluted serum, in portions, was then introduced into the sterile sac, special care being taken to keep the level of the fluid in the sac well below the end of the tube to which the sac was attached.

During each experiment, a negative pressure of 4 to 4.5 inches of mercury was maintained as far as possible; the receiver was kept in a container filled with ice, and the whole was covered with a black cloth. The results of several experiments with sacs indicated that the virus was not retained by collodion membranes of this type, as infectious filtrates were obtained in all cases where the sac did not burst due to mechanical pressure.

Filtration through agar.—The successful filtration of the virus through the collodion membrane led to some experiments with agar. Since an agar membrane seemed to be out of the question, recourse was had to the impregnation of a very porous Berkefeld bougie with agar, thus obtaining a filtering agar surface.

1. Contributions to Medical Research, 1903, Ann Arbor, Michigan, George Wahr. p. 390.

2. J. Infect. Dis. 1905, **2**: 14.

3. Personal communication. Technique later published by F. E. Bartell and M. VanLoo, 1924, J. Physiol. Chem. **28**: 161.

TABLE 2.—*Immunity to repeated doses of the virus in rats previously infected with* Sp. novyi.

Exp.	Rat no.	Interval in days	Number of injections	Result	Subinoculation
I	3.73.18	38*	1	0	
		12	2	0	
		8	3	0	
		5	4	0	
		9	5	0	
		9	6	‡9 days	‡2 days
		81†			
II	1.81	51*	1	0	
		6	2	0	
		7	3	0	
		5	4	0	
		9	5	0	
		9	6	0	
		7	7	‡16 days	‡1st 8 days ‡2nd 4 days ‡3rd 2 days
		95†			
III	4.102	72*	1	0	
		5	2	0	
		9	3	0	
		9	4	‡6 days	
		95†			
IV	5.102	104*	1	‡2 days	

Each dose was 0.25 cc of heart blood of rats dying of virus infection.
* This is the number of days that elapsed between the injection of *Sp. novyi* and the first injection of the virus blood.
† This is the total number of days from first to last injection.
‡ Indicates that animal died.

For this purpose, a Berkefeld bougie marked V (viel) was selected. It was first placed in a large test tube filled with water and steamed for 2 hours; it was then attached to the filtering cylinder and 500 cc of 2 percent aqueous agar, previously sterilized at 110 C for 20 minutes, were added. The cylinder was then attached to the filtering cylinder and 500 cc of 2% aqueous agar, previously sterilized at 110 C for 20 minutes, were added. The cylinder was then attached to an air tank and a pressure of 5 pounds was applied. When about 200 cc had passed through the filter, the tank was disconnected and the remainder of the agar in the cylinder was poured off. The inside of the filter was blown out to remove any agar which might clog the outlet. Then the cylinder with the attached filter was placed in an icebox overnight so as to allow the agar to solidify thoroughly.

The next day, 200 cc of a 1:100 dilution of the virulent serum were introduced into the cylinder and the filtration was started, first by gravity, which was too slow. Then an air pressure of 2½ pounds was applied and at intervals was raised to 5, 7½, and 10 pounds. A new receiver was attached each time and air pressure was increased. In this way, four separate filtrates (A to D) were obtained, and 10 cc of each one were injected intraperitoneally into rats. These died with typical symptoms, thus demonstrating the passage of the virus through the agar permeated filter. The results are given in table 2.

A second experiment made under like conditions gave essentially the same result. A third, a more exacting experiment, was carried out. In this, a new Berkefeld V filter and a 3% agar were employed. The filter was treated the same as in the first experiment but, after having been kept overnight in the icebox, it was given an extra coating of 3% agar. This was done by carefully rotating the treated bougie in a large test tube containing the 3% agar at 55 C, after which it was withdrawn and rotated in the air, in a horizontal position, until the agar solidified. Nine successive

coats were thus applied, after which the bougie was attached to the cylinder and the filtration was started. A total of 136 cc were collected in 8¼ hours. The filtrates, collected at 2½, 5, 10, and 15 pounds air pressure, were tested for the presence of the virus by injecting 10 cc of each into rats. The results were entirely negative. The control rat died in 62 hours of a typical infection.

Clearly, the additional 9 coatings of agar on the bougie, prepared as in the first two experiments, prevented the passage of the virus even under a pressure of 15 pounds.

MINIMUM LETHAL DOSE IN RATS

M.L.D.—The early attempts to determine the minimum lethal dose gave varying results. At first, the ordinary laboratory distilled water plus 0. 85% sodium chloride was used to make the necessary dilutions. Eventually, it was replaced by previously iced, glass-redistilled water.

Another factor was the blood of the donor rat. If it came from a rat which died in 48 hours, it probably contained more virus than that of a rat that died much later. Usually the blood was drawn from a rat that had just died and before rigor had set in. At other times, the dying rat was given a little ether before exposing the heart. It was possible for the ether to have an injurious action on the virus.

As a rule, the dilutions of the virus blood were made at once after the death of the donor, but, at times, this was impossible and the blood had to be iced for 3 to 30 hours. This procedure seemed to have an injurious action upon the virus as regards the M.L.D.

A third factor was speed in making the necessary dilutions and the immediate injection of the high dilutions into rats. With additional aid, this was usually accomplished in less than 10 minutes. A delay of 15 or more minutes was found to lower the infective dose. One cc of each dilution was injected intraperitoneally into each of the two rats.

Still another factor bearing upon the determination of the M.L.D. was the age and condition of the recipient rats. For that reason, young rats weighing about 100 g were used, but even then, the injection of the same dilution into 2 rats often caused the death of one and not of the other. There is a variation in the susceptibility of rats and this becomes more evident with dilutions of the order of 10^{-10} or 10^{-11}.

The net result of many early tests showed that the M.L.D. was 10^{-9} and that it might even be 10^{-10}. Some tests were also made with 10^{-12} and they were negative. However, three rats died after receiving a dose of 10^{-11} in three different tests, but these results were regarded at the time as accidental. Later experiments revealed the fact that the M.L.D. was actually 10^{-11}. This astounding value means that a cc of rat blood may contain at least 100 billion virus particles or 20 times the number of red corpuscles.

Presence of virus in the circulating blood.—Having established the pronounced infectivity of the agent, tests were planned to ascertain whether or not the virus persisted in the blood for some time after injection. One method of approach was to sacrifice the infected rat by bleeding from the heart while under ether. Rats were bled on the 14th or 28th day after the injection of a 10^{-11} dilution. Enough blood was obtained so that 1 cc could be injected into each of two recipients. The first test made after an interval of 14 days was successful, since the two recipients died of typical infection, but after an unusually long period of infection, namely 23 days. This proved the virus persisted in

the donor rat for 14 days, though in very small amounts, or in a weakened state. The other two tests made after an interval of 28 days were negative for 42 and 56 days.

Another method for determining the presence of the virus in rats that escaped death by the first dose of 1 cc of 10^{-11} dilution was to draw blood from the tail of the donor at certain intervals. In experiment I, the intervals were 14 and 28 days, and in experiment II, they were 7, 14, and 56 days.

In experiment I, after an interval of 14 days, one rat from each pair was "tailed" to two new rats for passage II, and it and its mate were injected with 1 cc of 10^{-8} virus dilution. It died in 4 or 5 days with typical symptoms, thus showing the absence of any immunity. After a second interval of 14 days, this procedure was repeated, resulting in passage III. After a further interval of 14 days, passage IV was made; then came passage V. The latter was made 70 days after the inoculation of the donor (rat 1.41).

The blood drawn at 14 days did not contain enough virus to kill either of the first recipients. Even passage II was negative. But passage III revealed the presence of the virus, though not in abundance, since the recipient, rat 1.119, died in 12 days after the last injection and 40 days from the start of passage I. This serves to show that the virus persisted in the blood of the donor for many days and that its recovery was not an easy matter.

Infectivity of red blood corpuscles.—The study of the M.L.D. of whole blood and of serum led to several attempts to ascertain whether or not the red blood cells were invaded by the virus.

For this purpose, the blood was centrifugated at 4000 r.p.m., after which the serum was removed with a pipette. The volume of the corpuscles was then measured, after which 100 volumes of salt solution were added and well mixed with the blood cells. The salt mixture was then centrifugated, after which the supernatant liquid was removed. The corpuscles were again diluted with salt solution, mixed and centrifugated. A third washing, in like manner, was then made.

The corpuscles were then diluted with salt solution and 1 cc of this suspension was injected into rat 18.5.32. The blood corpuscles were again washed three times with salt solution, after which they were diluted as before and 1 cc of the suspension was injected into rat 18.6.32. The washed red blood cells, after the first three washings, killed the rat in 61 hours; after the sixth washing, in 59 hours.

These and similar tests indicate that much of the virus present in the blood either adheres to or actually penetrates into the corpuscles.

IMMUNITY

It was observed during the study of the M.L.D. that immunity was not obtained by the injection of high dilutions of the virus, such as 10^{-11}, into clean young rats. In a few instances the rats did withstand a challenge dose of 10^{-8}, but when this was repeated, they died of the infection. Clearly, no immunity could thus be produced.

But, when as a matter of economy in maintaining the virus, it was decided to use old rats which had previously been infected with different strains of spirochetes, a surprising result was obtained. Some of these rats previously infected with *Spirochaeta novyi* showed a very high degree of immunity to the usual dose of 0.25 cc of the virus. This dose, ordinarily fatal in 2 to 4 days, was without effect as shown, in table 2.

It will be noted, in experiment I, that

rat 3.73 received its first injection of the virus 38 days after it had been inoculated with *Sp. novyi*. It then showed symptoms of a beginning infection but quickly recovered. The next four injections were without any visible effect. The sixth injection, made 81 days after the spirochete inoculation, however, caused death with typical symptoms. As a check, 0.25 cc of its heart blood was injected into a rat and produced typical death in 2 days.

In experiment II, it will be seen that rat 1.81 received its first injection of the virus 51 days after its inoculation with *Sp. novyi*. Further six successive injection of the virus were without effect. The 7th injection, however, caused typical death but after an unusually long period, viz., 16 days. To make sure that it died of virus infection, its blood was passed, in series, through three rats, which died in 8, 4, and 2 days, thus re-establishing the full virulence of the blood. Apparently, the virus by injection into rat 1.81 had been weakened by the antibodies present, more so than was the case in experiment I, where death was produced in 9 days, and a single passage of the heart's blood restored the virulence.

In experiment III, rat 4.10.2 received the first injection of the virus 72 days after injection with *Sp. novyi*. Three successive inoculations of the virus were without effect, but the 4th injection caused death in 6 days. The total time that elapsed between the injection of the spirochete and the 4th virus inoculation was 95 days, the same as in experiment II. Seemingly, the antibodies had been reduced in amount as compared with those in experiments I and II.

In experiment IV, rat 5.102 received the first and only injection of virus 104 days after inoculation with *Sp. novyi*. Typical death resulted in 2 days, and this normal death period may be taken to mean that either immunity had never existed or had been lost in 104 days. It may be added that this virus which killed in 2 days was used on the same day for the third injections in experiments I and II, and for the first injection in experiment III. In those three tests it had no effect.

When the first of these remarkable results was obtained it was looked upon as a nonspecific type of protection. Further study, however, made it clear that it was a real, active immunity due to a weak or attenuated virus. However, a long search for a stabilized attenuated virus, in association with *Spirochaeta muris*, proved unsuccessful.

At that time, the rats used in the laboratory were purchased from animal dealers from places as far south as Tennessee and St. Louis, Missouri. In some shipments, there were dead rats or deaths occurred shortly after arrival. Looking backward, it is perfectly clear that the virus was thus introduced into the laboratory as an inapparent infection. It probably was propagated by lice and other ectoparasites as a relatively mild infection.

Its association with *Spirochaeta kochi* (East African strain) led to such an increase in virulence that the inoculated rats died regularly in 2 or 3 days, and this fact led to the discovery of the virulent form of the virus. The other spirochetes did not have the aggressive action of *Sp. kochi* and, though delayed deaths occurred, no suspicion of the continued presence of the virus arose until active immunity was obtained, as shown in table 2.

Tests were then made with rats which had recovered from infection with *Spirochaeta duttoni* (West African origin), *Spirochaeta obermeiri* (Moscow origin), *Sp. muris* (Ann Arbor origin) and *Trypanosoma lewisi* (Ann Arbor ori-

gin). All of these showed active immunity on inoculation with 0.25 cc of the virus blood. This clearly showed that probably all of the old rats had an inapparent virus infection.

As has been stated, the serial passage of spirochetes was carried out by injecting new rats, every 2 or 3 days, either with the blood taken from the heart or from the tip of the tail. In the former procedure, the rat was sacrificed; in the latter it should have remained alive. But, since the inapparent virus infection was present, they died after a varying period of time. Thus, an examination of the *Sp. novyi* records, just preceding the recognition of the virulent virus in *Sp. kochi* rats for 3 months in 1909, probably at the start of the infection, revealed that 6 tailed rats died between 4 and 52 days. The record for *Sp. duttoni* was somewhat more complete and showed 13 deaths between 3 and 15 days in the same period of time.

The unrecognized presence of the virus in association with *Sp. novyi* almost caused the loss of this strain within a year after the virus was found in the *Sp. kochi* strain. It was saved by prompt passage through a white mouse. Two years later, however, it was lost during the vacation period. Fortunately, it was replaced by Dr. Noguchi, to whom it had been sent earlier.

CENTRIFUGATION

Quite naturally some attention was given to the centrifugation of the virus. A Danish centrifuge, having a maximum speed of 8000 r.p.m., was used for all the tests and was found to be inadequate. No attempt was made to centrifugate the undiluted serum. Instead, a 1:1000 dilution of the serum was used and this was centrifugated for 3, 6, or 12 hours. During this operation the top of the centrifuge was removed in order to avoid over-heating. The machine was run during the night, and the temperature of the room was thus kept at about 10 to 15 C.

TABLE 3.—*Centrifugation of the virus at 8000 r.p.m. Results of tests in rats of high dilutions of the top and bottom layers of the 1:1000 dilution made after centrifugation.*

Experiment time	Results in rats injected with 1 cc of:		
	Virus dilution	Top layer	Bottom layer
Experiment IV 3 hours	10^{-6}	*62 hrs.	0
	10^{-8}	*79 hrs.	*96 hrs.
	10^{-7}	*90 hrs.	*78 hrs.
Experiment V	10^{-6}	0	*85 hrs.
	10^{-8}	*108 hrs.	*85 hrs.
	10^{-7}	*85 hrs.	*108 hrs.
Experiment VI	10^{-6}	0	*120 hrs.
	10^{-8}	*77 hrs.	*120 hrs.
	10^{-7}	*72 hrs.	*72 hrs.

* Indicates that animal died.

After centrifugating, the top layer of the diluted virus was drawn off in a Pasteur pipette, and 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} dilutions were made and injected into rats. The bulk of the liquid in the centrifuge tube was removed in like manner, leaving the bottom layer, which was then used to make corresponding dilutions and injections. The results of three of these experiments are given in table 3. The table shows that the 10^{-9} dilution did not kill in 3 out of 6 tests. These negative results cannot be ascribed to centrifugation. They are probably due to variations in the susceptibility of the rat.

The conclusions drawn from these experiments was that the virus had not been sedimented by centrifugation at 8000 r.p.m. for 12 hours.

VIABILITY

The study of the viability of the virus was necessarily limited to that present in the blood of infected rats. The blood was drawn up into a heart pipette and defibrinated. The tube was then sealed with a rubber cap, or at times in a blast-lamp. The stock of infected blood was then kept in the cold room.

Viability in blood at about 9 C.—For these tests, the blood was kept in a cold room, the temperature of which varied somewhat from −4 to plus 10 C. The average temperature was 9 C. The refrigeration was effected with ice, and in addition, by means of a liquid carbonic acid machine which, however, worked intermittently. The tests began in January and ended toward the close of July, at which time the temperature of the cold room rose occasionally to 14 C. Had the material been kept at a strictly constant low temperature, it would undoubtedly have remained virulent for a much longer period. As it was, the virus remained infective for 149 days and was negative on the 182nd day.

Viability in blood at room temperature.—Tests were made at the same time with blood kept at room temperature (26 to 28 C) for 4, 7, 16, and 26 days. The virus tested on the 16th day killed in 89 hours, but not on the 26th day, when the rat recovered. Presence of bacteria in the blood stopped further tests.

Viability in blood at 37 C.—In a third set of tests, made at the same time as the preceding, the viral blood was kept in a capped tube at 37 C. At the start it killed in 70 hours. Tests made at the end of 20 and 68 hours resulted in death in 60 and 49 hours, respectively. The next test made at 118 hours (5 days) caused death in 12½ days (rat 4.79.17). A subinoculation was made with the blood of this rat, causing death in 6 days. A further test of the original blood made on the 8th day killed a rat in 11 days, and a subinoculation resulted in death in 36 hours. At this point, bacteria were found to be present in the original blood and further tests were discontinued. This experiment showed that the virus, when kept at 37 C, remained active for 5 and even 8 days though its virulence had decreased.

The general conclusions may be drawn that the virus retained its infectivity for 149 days when kept at low temperature (about 9 C), for 16 days at room temperature (26 to 28 C); and for 5 days and possibly 8 days at 37 C.

Viability of glycerinated blood virus.—For these tests the blood of several rats was pooled and an equal volume of glycerine previously sterilized at 110 C for 30 minutes was added. At first this mixture was placed in tubes and closed with rubber caps. In experiments II and III the tubes containing the blood mixture were sealed in a blast-lamp. But after they had been opened for the first test, they were closed with rubber caps. For each inoculation, 0.25 cc of the mixture was used.

Viability of glycerinated blood at about 9 C.—The temperature conditions were the same as has been given in the tests with whole blood. The results, likewise, were essentially the same. Whole blood was still infective at the end of 149 days but not at 182 days, while the glycerinated blood mixture caused death after 129 days and not after 272 days.

A very important fact, however, is brought out in experiments II and III. In these tests, the glycerin-blood mixtures were sealed in the blast-lamp instead of using rubber caps as in previous experiments. Surprisingly, the blood mixture in the sealed tubes remained infective for a much longer period of time, viz. 227 and 297 days, respectively. Unfortunately, the sealed tubes, after having been opened for the first test, were not resealed but were closed with a rubber cap. As a result, the virus soon died out in both experiments. It is highly probable that, had the tubes been resealed, the virus would have remained viable for a much longer period of time.

At all events, it became the custom in the laboratory to add glycerin to the blood drawn from the virus rats and to

seal the tube in the flame. The tubes thus sealed were kept in the cold room. This gave a reserve of glycerinated virus which could be counted upon in case of temporary loss of the virus. It was not known, however, that the virus would remain viable for more than a year.

The fortuitous recovery of the virus after a lapse of about 38 years demonstrated the remarkable viability of the virus under these conditions. It is possible that the virus retains its viability for a very long time when kept under decreased oxygen tension. It might also be pointed out that desiccation of droplets of the blood on the walls of the tubes may have been conducive to this prolonged viability.

Viability of the glycerinated blood at room temperature.—Tests were made at the same time with capped tubes kept at room temperature for 3, 13, 24, and 34 days. The virus killed a rat on the 3rd day in 96 hours, and on the 13th day in 92 hours, but it did not kill on the 24th and 34th day.

Viability at 37 C.—Similar tests were made at the same intervals, 3, 12, 24 and 34 days. The 3-day virus killed in 10 days, while the other 3 tests were negative.

It is evident from these tests that the virus maintains its viability the longest when kept at a low temperature, that of the cold room. Further, at 37 C the virus in whole blood retains its viability for about 11 days,—somewhat longer than that in the glycerinated blood.

THERMAL DEATH POINT

The first experiments to determine the thermal death point of the virus were made with whole blood. For this purpose, 0.25 to 0.50 cc of the blood was placed in a 12 mm test tube, which was then sealed in a blast-lamp. The tubes were then immersed in a water bath at a given temperature. The tests showed that the virus could survive in the blood for one hour at 45 C and at 50 C. Even after an exposure of 30 minutes at 55 C it killed in 52 hours; and at 60 C, for 15 minutes, it killed in 74 hours. The whole blood at this temperature soon coagulated solid and further tests were impossible. To avoid this difficulty in subsequent tests, the pooled blood was diluted with 1, 2, 4, and 9 parts of glass-distilled water.

TABLE 4.—*Thermal death-point of pooled virus blood diluted with glass distilled water.*

Temperature	Dilution	Time in minutes	Rat no.	Death in hours
50 C	1:1	5	1.17.21	*37
	1:1	15	3.17.21	*37
	1:2	5	2.17.21	*34
		15	4.18.21	*42
		30	1.18.21	*38
		45	2.18.21	*39
55 C	1:1	5	3.18.21	*45
		15	4.18.21	*50
		15	5.18.21	*45
	1:2	20	1.20.21	*42
		35	2.20.21	0†
		60	3.20.21	*94
60 C	1:1	15	1.19.21	*63
		30	2.19.21	*61
		45	3.19.21	*59

* Indicates that animal died.
† This was an old rat previously inoculated with *T. lewisi* and had become immune to the virus. It died 42 days later.

These dilutions were then taken up into narrow, drawn-out thin tubing and sealed in the flame. These capillaries were about 5 or 6 mm in diameter and 40 to 50 mm long. They were then immersed in a water bath at the desired temperature. After exposure to the heat, the tube was opened and about 0.5 cc of the diluted blood was injected into a rat.

It will be seen from table 4 that an exposure of 60 minutes at 55 C did not destroy the virus. Likewise, an exposure of 45 minutes at 60 C was without effect. In other tests made at 60 C a 1:4 dilution after 30 minutes killed in 85 hours; a 1:9 dilution after an exposure of 10 minutes killed in 59 hours. Unfortunately, no further tests were made to determine the killing time at 60 C.

ELIMINATION OF THE VIRUS

The extreme virulence of the blood as well as the condition of the diseased rat made it seem probable that the virus was freely eliminated during the course of the infection. This was all the more evident since the urine invariably had a more or less pronounced red color due to the presence of blood. Moreover, the eyelids and the nostrils not infrequently became stained a dark brown, showing that the serum exudate contained blood cells. It seemed certain, therefore, that these two secretions contained the virus.

To demonstrate the presence of the virus in the bloody urine, 0.5 cc of such urine was transferred from a rat, which died in 41 hours, by means of a fine capillary pipette, to a sterile tube and then injected into a white rat (1.102.19). The latter showed a typical infection in 36 hours and died in 56 hours.

The virus was demonstrated in like manner in the serous exudate of the eyes. For this purpose, a rat was selected which showed no evidence of blood in the exudate. A drop or two of sterile citrate solution was applied to each eye and then carefully removed with a drawn pipette. This procedure was repeated several times and the washings were then injected into a rat (2.102.19) which presented the usual eye and nose symptoms in 24 hours. The rat, when almost dead at the end of 55 hours, was bled to secure blood for another experiment.

The above tests proved the elimination of the virus in the secretions of the eye and kidney. No tests were made with the feces.

MODES OF INFECTION

Since the usual method of infection, by intraperitoneal injection of even very minute doses of the virulent blood, invariably resulted in fatal infection, it appeared very desirable to ascertain whether or not the disease could be conveyed by direct feeding, by contact of the virus with the mucous membranes of the eye and throat and by ordinary contact or association, including lice.

Feeding experiments.—The first three tests were made by feeding the heart, liver and spleen of infected rats. In two of the tests, the rats were deprived of food for a day in order to insure rapid ingestion of the diseased organs; in the third set no such precaution was taken. The material was eaten by the rats within 15 minutes.

Test 1—Rat 4.71.18 was fed on organs of rat 5.66 that died in 44 hours. It was found dead 86 hours later.

Rat 5.71.18, fed on same material, died in 88 hours.

A control rat, 6.71.18, received 0.25 cc of the heart-blood of the rat whose organs were fed to the above rats. It died in 52 hours.

Test 2—Rat 7.71.18 was fed the organs of rat 6.71. It died in 73 hours.

Rat 8.71.18 was fed the same material and died in 84 hours.

Test 3—Rat 3.75.18 was fed the organs of rat 8.74.18 that died in 29 hours. It died in 91 hours.

Rat 4.75.18 was fed on the same material and died in 60 hours.

A control rat, 7.75.18, received 0.25 cc of the heart blood of 8.74.18 and died in 37 hours.

It will be seen from the above that the feeding of diseased organs, rich in virus, is fatal in from 3 to 4 days.

In order to ascertain the effect of feeding a relatively small amount of virus, the blood of a rat that died in 69 hours after inoculation was mixed with an equal volume of sterile glycerin. One drop of the mixture was placed on a piece of soft bread and fed to a rat. Five rats were thus fed. Two of the rats died in 94 and 98 hours respectively, while three showed no ill effect. The three surviving rats were given a second feeding of fresh glycerinated virus, 30

days later. This time one died in 80 hours, 1 in 8 days and the third survived. The latter was given, 16 days after the second feeding, an injection of 0.25 cc of virulent blood and died in 60 hours, thus showing absence of immunity.

Infection through the mucous membrane of throat.—In order to test the infectivity of the virus when applied to the mucous membranes, the rats were immobilized by means of a leather jacket, after which the throat of each animal was touched, several times in the course of half an hour, with a cotton swab dipped in virulent blood. Five rats thus tested with three different bloods, died in 70, 73, 77, 90 and 90 hours, respectively.

Application of the virus to the eye.—Three tests, each with two rats, were made. The animals were immobilized as above and one drop of virulent blood was dropped into each eye. After 30 minutes the rats were released. Three different specimens of virulent blood were used. Five of the rats died in 73, 100, 100, 102 and 120 hours, respectively and only one escaped infection.

Contact infection.—One experiment was made to ascertain whether healthy rats could acquire the virus infection by mere contact or association with infected rats.

For this purpose, 6 half-grown white rats were placed in a cage and an infected rat was also placed therein. When this rat was about to die, it was removed and, as soon as possible, it was replaced by another infected rat. This was repeated, from time to time, so that in the first 24 days, 11 infected rats were introduced into the cage. On the 32nd day of the experiment, rat 1.107.22, the first of the 6 clean rats, died of typical virus infection. On the 47th day, 2 infected rats (nos. 12 and 13) were placed in the cage with the remaining 5 clean rats. On the 49th day, 4 more infected rats (nos. 14 to 17) were placed in the cage. Of the five remaining rats, two (2.107.22; 3.107.33) developed typical infection and died on the 50th and 51st day of the experiment and a third rat (4.107.22) died on the 76th day. The two remaining clean rats, after exposure to 17 infected rats over a period of 82 days, were each given an injection of 1 cc of 10^{-8} dilution of the virus blood. They both died of typical infection in 120 hours. A control rat (5.60.23) was given the same dose and died in 105 hours.

The four contact infections were probably due to mouse or flea transmission. The possibility of contamination with the eye secretion, or bloody urine, or even the feces is not excluded. No devouring of the infected rats took place because of the prompt removal of the dying rats.

ACTION OF THE VIRUS ON OTHER ANIMALS

The virulent rat blood, injected intraperitoneally in a dose of 0.25 to 1.0 cc, was without effect in the following animals: 2 young hogs, 2 young chickens 2 pigeons, a dog, a cat, rabbits, guinea pigs and house mice.

White mice.—The virus was not tested on suckling white mice. In general, the full grown white mouse appeared to be resistant to the usual dose of 0.25 cc of the virulent blood. Occasionally, however, the mice died, and this was true especially when a second injection was given a few days after the first, and where the mice were of a crossed breed.

Thus, a black and white mouse (1.90 .17) after an injection of 0.25 cc died in 93 hours, with typical symptoms. A drop of its blood injected into a white rat caused death in 74 hours. Another black mouse (2.90.17) which received a similar injection as the preceding one

was apparently unaffected. But, when given a second injection 8 days later, it died in 36 hours.

Later experiments with pure white mice showed that an inapparent infection does occur. Thus, a mouse (5.27.19) was bled on the 7th day, after it had been given an injection of the rat virus, and 0.25 cc of its blood was injected into a rat (1.28.19). The latter died in 88 hours with typical symptoms, thus proving that the virus was present in the blood of the mouse on the 7th day. However, a second mouse (6.27.19) injected at the same time with the same rat blood was bled on the 14th day and its blood was injected into a rat (2.28.19). The latter showed no effect, thus proving that the virus had disappeared from the blood of the mouse in 14 days. A third mouse (7.27.19), likewise injected at the same time with the rat virus as the preceding two mice, showed no ill effects for 44 days. It was then given a second injection and 6 days later its blood was injected into a rat (3.28.19) which showed no sign of infection. Apparently, the mouse had developed an immunity which enabled it to destroy the virus of the second injection. Three other mice (9 to 11.27.19) were injected on the same day as the preceding three mice but with the virus from another rat. No ill effects were observable. On the 44th day, they were given a second injection with likewise negative results.

It is evident from these experiments that the white mouse, when injected with the virus, may develop an inapparent infection which soon results in complete immunity.

Symptoms.—The wild rat and the white rat, half to full grown, were the only animals really susceptible to the virus. No tests were made with suckling or very young rats. The first signs of infection were an indifference to feeding and a roughening of the hair. The rat withdrew into a corner and did not respond to prodding. It was unable to walk and tottered. The eyes appeared glassy. The eyelids became glued with a serous exudate which was sometimes colored with dark or black blood, and the nostrils at times also showed dark crusts. A drop of bloody urine was often seen at the urethral opening. Finally, there was usually a convulsive leap; the rat fell down on its side and its legs twitched a few times. Death followed. Occasionally, there was an early excited or wild stage with convulsive seizure. The rat jumped up blindly and hit the wire top of the cage; on falling down, it vigorously pawed the shavings on the bottom. It was clear from these symptoms that the brain was involved and several examinations for Negri-like bodies were made by Dr. J. G. Cumming with negative results. Obviously, it was an encephalitis though it was not recognized as such at that time.

When blood was drawn from the heart, at or just before death, into a heart pipette, the yield was small. It was dark in color and coagulated very slowly. This small yield of slowly coagulating dark blood was very characteristic of the infection. Invariably, with the usual dose of 0.25 cc of the heart blood, death occurred in 36 to 72 hours, and with smaller doses it was delayed for 6 to 8 or 12 days. After death, the stomach was found empty and distended and the bladder was usually full of bloody urine (hemoglobinuria?).

SUMMARY

A virus was introduced into the laboratory by naturally infected white rats purchased from animal dealers. Its presence was unrecognized for some time, not until its virulence had greatly increased. Lice and other ectoparasites were undoubtedly the natural means of

transmission. The serial passage of spirochetes and trypanosomes in rats served to continue the virus infection. The symptoms were those of an encephalitis.

The virus was maintained in rats for a number of years until it was apparently lost. Recently, after a lapse of more than three decades, its recovery has been effected. That fact has prompted the publication of some of the old data regarding the virus.

RECOVERY OF THE NOVY RAT VIRUS

RUSSELL T. JORDAN,* W. J. NUNGESTER, AND W. S. PRESTON
From the Department of Bacteriology, University of Michigan, Ann Arbor, Michigan

Approximately 40 years ago, Dr. F. G. Novy, working with rats infected with spirochetes of relapsing fever, noted that Berkefeld filtrates of the blood of such rats produced a fatal infection in normal rats inoculated intraperitoneally.[1] Further studies by Dr. Novy and his colleagues of the filtrable agent showed that rats were susceptible to high dilutions of the infectious agent. Since a detailed account of the original isolation from rats is given elsewhere,[1] a further history of this agent will not be presented.

In the course of cleaning out a laboratory, 25 hermetically sealed tubes were found. The labels on some of these tubes could be read. It was apparent that they contained glycerinated blood of rats infected with the Novy rat virus in the period between 1914 and 1918. The tubes, when found, were at room temperature. It is believed that at an earlier period they had been kept in a refrigerator. Although the first reaction was to discard this material, it was decided to pool the contents of the tubes and inject the material into rats just to be sure that the long lost "Novy" virus was not present. The unexpected results are reported in this paper.

EXPERIMENTAL

Material and methods.—The "stored" virus consisted of 25 hermetically sealed glass tubes, each containing 4 to 6 ml glycerinated (1:1) blood from rats infected with the Novy virus. The tubes were divided into groups of 5 each, washed with soap and cold water, and immersed in cold 70% ethanol for 1 hour. The contents of the 5 tubes of each group were pooled and ground in a mortar, using portions of the glass tube on which particles of blood had dried during the sealing process as abrasive. An aliquot portion of the suspension was cultured on blood agar and in thioglycollate broth, with negative results.

Animals used.—All rats used were of an albino strain raised in the Department of Pathology at the University Medical School, and were 3- to 4-week-old males weighing from 45 to 60 g. The mice used in the experiments, obtained from a local source, were of a Webster strain, and 3 to 4 weeks old.

Inoculations.—Since the original isolation of the Novy virus was made during the serial passage of relapsing fever spirochetes,[1] it was considered advisable to inoculate the test animals with a spirochete prior to the inoculation of the stored blood, as it was believed that the spirochete might in some way alter the resistance of the rats and thereby render them more susceptible to the stored virus.

Twenty-five rats were divided into 5 groups. Each rat was injected intraperitoneally with 1.0 ml of heparinized *Spirochaeta novyi* infected rat blood containing approximately 25 spirochetes per oil immersion dark field. This was followed in 24 hours by 1.0 ml intraperitoneally and 0.05 ml intracerebrally of undiluted stored blood from one of the 5 pools suspected of containing the Novy rat virus. Despite the fact that some of the animals showed convulsions following intracerebral inoculation of

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1. F. G. Novy, Perkins, W. A., Chambers, R. and DeKruif, P. H. 1953, The rat virus. J. Infect. Dis. **93**: 111–123.

TABLE 1.—*Fate of rats following injection of stored blood.*

Rat	Group				Controls
	1	2	4	5	
1	6d	5i	5i	5	S
2	3i	5i	15d	S	S
3	11d	8d	3d	4i	S
4	6d	8d	S	5i	S
5	S	S	S	S	S

Groups 3 discarded due to bacterial contamination.
d, day of death.
i, signs of infection.
S, survived.

the ground glass blood mixture, all survived the injection.

Control animals of the same age and weight received the same dose of *Spirochaeta novyi* infected blood intraperitoneally and 0.05 ml of a mixture prepared from normal rat blood and ground glass intracerebrally.

Serial passage of blood from rats showing signs of infection following injection of the blood mixture.—Results of the first passage appear in table 1.

From table 1 it may be seen that in group 1, one animal showed signs of infection as early as the 3rd day, and 2 rats had died by the 6th day following inoculation, whereas all control animals survived. Reference to group 2 shows that on the 5th day, 2 rats were showing signs of infection, and in group 5, one rat was sick on the 4th day. It should be pointed out that in group 4 the incubation period of one rat was prolonged for 21 days, and that at least one rat survived in each of the 4 groups used in the experiment.

Signs of infection consisted of ruffled fur, hunched backs, listlessness and apparent lack of interest in food and water. Encrustations of blood were common findings around the nose and both front paws. A characteristic hematuria in rats dead of infection with the Novy virus has been described by Dr. Novy.[1] Hematuria in certain of the rats following intraperitoneal injection of the recently isolated virus has been inconstantly observed. One or two days after the first signs of infection, animals become prostrate and death rapidly ensues.

Both rats in group 2 showing signs of infection were lightly etherized and 2.0 ml of blood were removed from each animal by cardiac puncture. The heparinized blood from rats 1 and 2 was then pooled, gently shaken, and 0.1 ml placed on a blood agar plate and incubated at 37 C for 48 hours. No growth was obtained. The rats were then sacrificed and the brains were aseptically removed, pooled and ground in a mortar, using alundum as an abrasive and enough cold sterile saline to make a 10% suspension. The suspension was then centrifuged at approximately 1500 r.p.m. for 5 minutes to sediment gross particles. The supernatant fluid was removed, and 0.1 ml was cultured on blood agar at 37 C for 48 hours. No evidence of bacterial growth was noted.

Each of 5 rats, 3 to 4 weeks old and weighing approximately 50 g, were injected intraperitoneally with 1.0 ml of the heparinized blood from rats 1 and 2. Darkfield examination of this blood prior to injection showed the presence of several spirochetal forms per field. Each of the rats was then injected intracerebrally with 0.05 ml of the 10% brain suspensions from rats 1 and 2 prepared above.

Serial passage was made five times, pooling portions of blood and brains of each of 2 rats showing signs of infection. Prior to each passage, the blood collected by cardiac puncture was examined for the presence of spirochetes by darkfield examination and cultured for possible bacterial contamination.

Following each passage of blood and brain suspension, 3 or 4 of the rats from each group became prostrate and died of infection, or were sacrificed for further passage material.

Filtration experiments.—Having demonstrated that injection of blood and brain suspensions from spirochete-infected rats was uniformly fatal for normal rats of the same age and strain, an experiment was designed to determine whether death of the animals was due to the presence of the spirochetes or to a virus which might have been present in the original stored, glycerinated blood.

On the 5th serial passage, 2 rats which 5 days previously had received 1.0 ml of undiluted rat blood containing spirochetes and 0.05 ml of pooled brain suspension and which had developed signs of infection, were sacrificed by bleeding from the heart. Approximately 2.0 ml of heart's blood were collected from each animal and pooled. Darkfield examination of the heparinized blood showed the presence of several spirochetes per field. The blood was then centrifuged at 1500 r.p.m. for 5 minutes. The plasma was removed and passed through a Berkefeld (W) filter with the aid of suction. The filtrate showed no spirochetes on darkfield examination, and 0.1 ml of the filtrate produced no growth on a blood agar plate. However, approximately 1.0 ml of the spirochete-free filtrate injected intraperitoneally into each of three 40-g rats produced typical signs of infection in 2 of the rats as early as the 4th day, and one animal died on the 5th day. Darkfield examination of heart's blood from these animals failed to demonstrate the presence of spirochetes.

The infectious agent also passed a Seitz EK filter pad and produced death of 3- to 4-week-old rats injected intraperitoneally with the filtrate. Studies with gradacol membranes have shown the probable size of the virus to be in the range of 20 to 25 mμ.

Titration of the infectious agent in rats by intraperitoneal and intracerebral routes.—Two rats injected intraperitoneally with infected blood of the 25th serial passage developed signs of infection, and were bled to death by cardiac puncture. Approximately 1.5 ml of blood were obtained from each animal, and were pooled in a tube containing a small amount of heparin. Decimal dilutions of this blood ranging from 1×10^{-1} to 1×10^{-10} were made in cold sterile saline. Five rats, each receiving 1.0 ml intraperitoneally, were used to test each dilution. Test animals which survived were observed for 60 days.

The LD_{50} titer[2] of the infected rat blood injected intraperitoneally was $1\times10^{-7.8}$. However, it should be pointed out that the range of the incubation periods in rats which succumbed to infection, following intraperitoneal inoculation of the same dilution of infected blood, was from 3 to 27 days. Certain of the rats which were injected with dilutions of 1×10^{-7} showed signs of beginning infection but later recovered.

On the other hand, the intracerebral titration of infected rat brain suspensions in 40-g rats following the 25th intracerebral passage gave an LD_{50} titer of $1\times10^{-10.8}$. The incubation period of animals which died of infection following intracerebral inoculation of infected brain suspensions ranged from 18 hours to 6 days. It is interesting to note that all of the rats which showed signs of infection following intracerebral inoculation eventually died, whereas certain of the rats which had received an intraperitoneal injection of infected blood and became infected did not always succumb. In general, it has been observed that the intracerebral route of inoculation produces a fulminating infection which is uniformly fatal for rats

2. Reed, L. J. and Muench, H. 1938, A simple method of estimating fifty per cent end points. Am. J. Hyg. **27**: 493–497.

TABLE 2.—*Neutralization tests using the Novy virus and various hyperimmune serums.*

Antiserums tested	Dilution of virus tested						LD_{50} titer	Neutralization index
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}		
Venezuelan Equine[1]	5/5*	5/5	5/5	3/5	0/5	—	7.1	10.0
Western Equine	5/5	5/5	5/5	4/5	0/5	—	6.8	15.0
Eastern Equine	5/5	5/5	3/5	2/5	0/5	—	6.5	52.0
Semliki Forest	5/5	5/5	5/5	5/5	—	—	0	
Mengo	5/5	5/5	5/5	5/5	—	—	0	
Rabbit serum control			5/5	4/5	3/5	0/5	8.0	

[1] The Venezuelan antiserum was produced in a guinea pig, other antisera used were produced in monkeys.
* The denominator indicates the number of animals inoculated and the numerator indicates the number of deaths.

of all ages, whereas by the intraperitoneal route of inoculation, rats appear to vary in susceptibility to the lethal effect of the virus.

Adaptation of the virus to the albino mouse.—Since the albino rat had been the only experimental animal used for passage of the infectious agent, it was considered of interest to determine the adaptability of the agent to the albino Swiss mouse. Three- to four-week-old mice ranging from 7 to 10 g. were used. Each of 5 mice was injected intraperitoneally with 1.0 ml and intracerebrally with 0.05 ml of a 20 % suspension of infected rat brains. Only 3 of the animals showed any evidence of infection. Serial passage of brain suspension and blood obtained from mice showing mild signs of infection was continued for 5 passages. By the 5th passage, signs of infection began to increase in severity, and by the 7th passage, infected brain suspensions injected intracerebrally in mice produced a uniformly fatal disease in 48 to 72 hours.

Neutralization tests.—To investigate the possible relationships of the Novy virus to known viral agents up to 50 mμ particle size, neutralization tests were performed. The technique used for the tests was that recommended by the Neurotropic Virus Commission in 1942.[3] Hyperimmune monkey serums used in these tests were obtained through the courtesy of Dr. John Bugher and Dr. Kenneth C. Smithburn and included western equine encephalomyelitis (W.E.E.), eastern equine encephalomyelitis (E.E.E.), Semliki Forest and Mengo antiserums. Venezuelan equine encephlaomyelitis (V.E.E.) antiserum, produced in a guinea pig, was kindly supplied by Dr. Roy Fothergill.

Infected mouse brains were ground in a mortar with alundum and sufficient inactivated, undiluted rabbit serum to make a 10% suspension. The suspension was then centrifiged at 2000 r.p.m. for 5 minutes and the supernatant fluid removed and distributed in 2.0 ml amounts in screw-capped vials. These were quickly frozen by means of a mixture of dry ice and alcohol, and then stored at −60 C until needed.

In mice the intracerebral LD_{50} titer of the above stored virus suspension was determined to be $1 \times 10^{-8.0}$. Tenfold serial dilutions of the virus were prepared in an ice bath, using 10% inactivated rabbit serum in saline as diluent. From each of the virus dilutions to be tested, 0.2 ml was placed into each of 2 tubes. To one series of tubes (control) was added 0.2 ml of 10% normal inactivated rabbit serum. To the test series 0.2 ml of the hyperimmune serum to be tested was added. The tubes were incubated in a 37 C water bath for 2 hours and placed in an ice bath prior to

3. Paul, J. R. 1944, The filtrable viruses in laboratory methods of the United States Army, edited by Simmons, J. S. and Gentzkow, C. J., Philadelphia, Lea and Febiger, pp. 579–600.

injection. Five mice were used for each dilution tested. The control series consisted of virus dilutions from 10^{-6} to 10^{-9} and test serums of dilutions from 10^{-4} to 10^{-7}.

Table 2 is a summary of the neutralization tests. The results indicate that there was little or no neutralization by any of the serums tested, with one exception. Some evidence pointed to a slight relationship between E.E.E. and the Novy virus. On the other hand, the tests gave little or no indication of a relationship between the Novy virus and the viruses W.E.E., Mengo, V.E.E. or Semliki Forest.

Control experiment.—The passage of blood or tissue suspensions in series from one animal to another is a procedure which must be undertaken with a full realization of the many possible pitfalls. The presence of latent infectious agents which are made active by repeated animal passage, as well as the possibility of an active infection in one or more animals, must be considered. In order to investigate the possibility of a latent infectious agent in the stock of laboratory rats used in these experiments, the following procedure was carried out. Twenty-five rats of the same age, sex and weight as those used in the previous experiments were divided into 5 groups of 5 animals each. They were injected intraperitoneally with 1.0 ml of heparinized *Sp. novyi* infected rat blood containing approximately 30 spirochetes per darkfield. The spirochetes used were from the same source and strain employed in the experiment from which the Novy virus was re-isolated. In order to produce an irritation comparable to that produced by the administration of old blood and ground glass, the following technique was employed. Twenty ml of citrated human blood, which had been collected approximately 7 months before and stored in the refrigerator, was placed in a mortar containing glass particles and the mixture ground. Each of the 25 rats, which had received the spirochete-infected rat blood 24 hours previously, was inoculated with the citrated human blood-ground glass mixture, 0.05 ml intracerebrally and 1.0 ml intraperitoneally. The rats were observed for 60 days.

Four days following intraperitoneal and intracerebral inoculation of the blood-glass mixture none of the rats were showing any evidence of infection. Two rats from group 1 were selected at random and exsanguinated by cardiac puncture. Approximately 2.0 ml of heparinized blood were obtained, pooled and examined by darkfield illumination for the presence of spirochetes. Several spirochetes were noted per field. The brains of the 2 sacrificed rats were aseptically removed, and a 10% suspension was prepared in cold sterile saline. Each of 3 normal rats was then inoculated intracerebrally and intraperitoneally as described in the original isolation experiment. The collection of blood, the preparation of brain suspension, and the inoculation of normal rats was conducted in exactly the same manner with the four remaining groups. Even though the above procedure was repeated through 5 serial passages, none of the inoculated rats showed any apparent evidence of virus infection.

SUMMARY

1. From the data presented, it appears that a filtrable infectious agent has been recovered from virus-infected rat blood collected and stored by Dr. F. G. Novy approximately 35 years ago. Moreover, spirochete-free heart's blood passed in series continued to produce a fatal infection in rats subsequently injected.

2. The infectious agent which has been isolated produces a fatal infection

in young rats and mice when injected by either the intracerebral or intraperitoneal routes.

3. The virus passes Berkefeld W and Seitz EK filters and is estimated by gradacol filtration to have an approximate size of 20 mμ.

4. Neutralization tests, using serial dilutions of the Novy virus and hyperimmune monkey and guinea pig serums, indicated that there is no relationship between the Novy agent and W. E. E., Mengo, V.E.E. or Semliki Forest viruses.

5. Neutralization tests did, however, point to a possible relationship between E.E.E. and the Novy virus, although this relationship is not marked.

THE INFLUENCE OF ADRENAL CORTICAL EXTRACT ON ANTIBODY FORMATION, INCLUDING THE NONSPECIFIC ANAMNESTIC REACTION IN THE RABBIT

ABRAM B. STAVITSKY

From the Department of Microbiology, School of Medicine, Western Reserve University, Cleveland 6, Ohio

There have been many attempts to determine the effect of adrenal cortical hormones on the resistance of the host to various noxious and infectious agents.[1] These studies generally have shown a beneficial effect of these hormones on the host's reactivity to harmful agents,[1,2] but have not provided an understanding of the mechanisms involved. In 1945, Dougherty and White reported the results of studies on the physiological functions of these hormones.[3–5] They noted that the injection of adrenal cortical steroids or pituitary ACTH into mice, rats, and rabbits was followed in several hours by a reduction in the number of circulating lymphocytes, presumably as a result of the dissolution of these cells in the lymphoid organs. A similar effect could be produced upon subjection of these animals to various stressful stimuli which caused increased adrenal cortical secretion. During the destruction of the lymphocytes portions of their cytoplasm were shed into the blood, producing an increase in the total serum protein. When the lymphocytes of immunized animals were found to contain antibody protein, the effect of the injection of adrenal cortical hormones on the level of antibody in the serum was determined. It was found that the hormones had striking effects on increasing the level of serum antibody. Injection of hormones during immunization also brought about an increase of the antibody titer. In hyperimmunized rabbits, a single injection of hormone resulted in a rise in antibody within 6 hours. Previously immunized rabbits, which were allowed to lose their antibody titer over a period of months and then given hormone, showed with a few hours a striking rise in titer much greater than the rise caused by nonspecific protein or specific antigen alone.

It was concluded from these findings that adrenal cortical hormones exerted an important influence on the circulating antibody titer through their ability to cause the lysis of lymphocytes with the release of cytoplasmic proteins, including immune γ globulin in immunized animals. Moreover, when stimulated, the adrenal cortex could bring about release of antibody from cells in previously immunized animals which did not currently have any circulating antibody. It appeared that the increased adrenal cortical secretion might be the basic mechanism of the anamnestic reaction which had been noted before[6–8]

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as a consequence of the application of a variety of nonspecific stimuli to previously immunized animals and man.

In view of the fundamental nature of these studies, there have been many attempts to confirm them. Varying results have been obtained, including considerable inhibition,[9,10] slight or no effect,[11–14] and enhancement of antibody formation.[15,16]

The present experiments were initiated as part of a broader study[17,18] of the basic mechanisms of antibody formation. In the course of this study it became desirable to produce the anamnestic response by the injection of adrenal cortical extract. It was considered that the negative results of previous studies might have been due to failure of accurate reproduction of the conditions of the original experiments. Investigators had deviated from the original experiments by employing different animals, amounts of hormone, hormone preparations, times of injection, intervals between immunization and injection of hormone, and, finally, different methods of measuring the amount of antibody. Any of these deviations might affect the final result. Therefore, some of the original experiments of Chase, Dougherty and White were repeated by following the descriptions in their original papers. When attempts to confirm some of the original observations were unsuccessful, experiments were done using various antigens, routes of injection, immunization schedules, quantitative methods for determining antibody protein, as well as another method of producing the anamnestic reaction. In confirmation of previous work,[9,10] these experiments generally have resulted in the inhibition of antibody formation by the injection of adrenal cortical extract.

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MATERIALS AND METHODS*

Antigens.—The antigenic preparations employed included: (1) egg albumin (EA), (2) *Shigella paradysenteriae*, type Z (SH), (3) sheep erythrocytes (RBC), (4) diphtheria toxoid, alum-precipitated, 40 Lf per ml, (5) tetanus toxoid, alum-precipitated, 20 Lf per ml. All preparations were dissolved in saline solution for injection.

Animals.—Heterozygous albino rabbits weighing about 6 pounds were used. No segregation according to sex was made, as it did not appear to be an important factor in the study.

Hormone preparation.—Adrenal cortex extract (ACE) of the Upjohn Company was used throughout this study. Ten percent "aqueous alcohol," which is the solvent used for this product, often was employed for injections into control animals. Subcutaneous injection of 5 or 10 ml of the ACE into rabbits consistently led within 3 to 6 hours to the production of a lymphocytopenia.

Blood counts.—The white cells were counted in the usual manner. The slides for the differential count were stained with Wright's stain; 100 cells were counted.

Precipitation tests.—In the titration by dilution of antiserum, doubling dilutions of the serum

* The author is grateful to the following organizations for generous gifts of materials: Armour Laboratories—egg albumin; Lederle Laboratories—diphtheria toxoid; Upjohn Company—aqueous adrenal cortex extract and 10% "aqueous alcohol."

were made to 1:20,480, and the limiting dilution of antigen which gave a precipitate was added to each tube.[19] The highest dilution showing visible precipitation after storage at 37 C for 2 hours and in the refrigerator overnight was called the highest titer.

The quantitative precipitin technique[20] was employed with slight modification. Two-tenths ml of antiserum was mixed with varying concentrations of the antigen. The mixture was then incubated at 37 C for 1 to 1½ hours and in the refrigerator for 1 to 2 days. The resulting precipitate was washed twice in ice-cold saline and analyzed by the Biuret-Phenol colorimetric procedure.[21] The latter method permitted accurate, reproducible determinations of as little as 20 μg of protein. The results obtained by this technique were within 5% of the analytical data secured by the Kjeldahl method.

Agglutination tests.—Agglutination tests were used routinely for the titration of sheep hemagglutinins. The serums were diluted by the doubling-dilution method and 0.5 ml of a 2.5% suspension of fresh washed cells was added to each tube. The tubes were incubated in a water bath at 37 C for 1 hour, put in the refrigerator overnight and then read.

In the bacterial agglutination tests, 0.5 ml of doubling dilutions of antiserum was mixed with 0.5 ml of heat-killed bacterial antigen. The mixtures were spun immediately for 10 minutes at 1500 r.p.m. The reaction read at this time compared very closely with that observed following incubation of the cell-serum mixtures at 37 C or 56 C and storage overnight in the refrigerator. The centrifuged cell-serum mixtures could readily be distinguished from centrifuged cell mixtures, which resuspended readily into a uniformly turbid suspension compared to the clumps of agglutinated organisms when cell-antiserum mixtures were spun. The antiserums were always inactivated at 56 C for 30 minutes since it was found that complement interfered with the agglutination of the bacteria by low titered serums.

The protein hemagglutination tests were carried out with some modifications of the findings of Boyden[22] on the coating of tannic acid-treated erythrocytes with foreign proteins and agglutination of these cells by the specific antiprotein serums. It was found that egg albumin and diphtheria toxoid could be coated on sheep erythrocytes in this manner and that these cells were specifically agglutinated by the homologous antiserums. This test was very sensitive, permitting the detection of about 0.015 μg of antibody protein. The serums had to be inactivated at 56 C for 30 minutes and adsorbed with sheep red blood cells in order to eliminate false positive reactions.

Antitoxin determinations.—Antitoxin was determined by the rabbit intracutaneous method of Fraser.[23]

Bleeding and complement titrations were conducted as described in a previous paper.[24] The complement titers are expressed in terms of ml of 1:10 dilution of rabbit serum required to hemolyze 50% of sensitized sheep red blood cells. Therefore, the *larger the figure the lower the concentration of complement in the serum and vice versa.*

EXPERIMENTAL

Influence of ACE on the production of precipitins to ovalbumin

This experiment was designed to repeat the work of Chase, Dougherty, and White.[5] In addition to the titration of precipitins by the antiserum dilution method employed by these authors, two additional methods were employed. First, the earliest appearance of antibody was detected by determining the complement titer of the blood serum before and 30 and 60 minutes after each injection of antigen. This determination was based on the previous finding[24] that the injection of antigen into an immunized animal was followed by a prompt and considerable reduction in the level of complement, presumably due to in vivo fixation of complement by the antigen-antibody complex. The second method involved the use of the quantitative precipitin technique.[20] The measurements of antibody were made

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TABLE 1.—*Effect of ACE on precipitin production to ovalbumin.*

Rabbit no.	Date	Antiserum titer	AB—N	C′ titer			Lymphocytes/cu mm				
			ml	0	30	60 min.	0	3	6	9	24 hr.
							in thousands				
41	1-14			0.88	0.92	1.00	69	27	51	62	74
ACE	1-17			0.95	0.95	0.90	78	34	38	73	82
	1-21			0.80	0.82	0.84	70	28	24	70	72
	1-25			0.95	1.90	0.92	70	24	30	73	72
	2-2	12	0.38								
	3-2	48	0.51								
45	1-14			1.40	1.60	1.90	64	21	22	48	71
ACE	1-17			0.90	1.20	0.95					
	1-21			0.85	0.94	0.94					
	1-25			0.90	0.90	0.96					
	2-2	12	0.36	0.85	3.60	1.80					
	3-2	96	0.64								
49	1-14						74	21	28	34	78
ACE	1-17			1.6	1.4	1.3					
	1-21			1.8	1.4	2.0					
	1-25			1.4	3.2	1.8					
	2-2	24	0.39								
	3-2	96	0.72								
42	1-14			0.75	.75	0.80	62	70	100	40	56
Control	1-17			0.70	1.80	0.90	58	40	60	71	54
	2-2	24	0.79								
	3-2	48	1.40								
46	1-14						61	61	72	58	61
Control	1-17			1.0	2.4	1.5	68	72	54	62	68
	2-2	24	0.20								
	3-2	96	1.0								
50	1-14			0.60	0.65	0.68	48	40	28	44	47
Control	1-17			0.60	0.90	0.70	42	38	44	47	49
	1-21			0.70	1.90	0.75	42	21	48	54	41
	2-2	24	2.50								
	3-2	48	2.75								

Antiserum titer—take reciprocal of figure.
AB—antibody; C′—complement titer = ml 1:10 rabbit serum to hemolyze 50% sensitized sheep red blood cells.
Immunization schedule: #41, 45, 49—1 ml 1% EA 1/14, 1/17, 1/25, 2/5, 2/8, 2/25; 4 ml 1% EA 1/21 and 2/12; 2 ml 1% EA 2/17 plus 3 ml ACE subcutaneously each time antigen given. #42, 46, 50 same as previous rabbits but ACE "diluent" substituted for hormone.

on the serums collected after about 2 and 6 weeks of immunization, because in the original study[5] at these times there appeared to be much more antibody in the serum of the hormone-treated than the control rabbits. Total white blood cell and differential counts were done often because of the lability of the blood picture in the rabbit and to exclude the possibility that handling and bleeding alone might produce an increased adrenal cortical secretion in the control animals. Previous experiments[25] had shown that ACE did not exert any significant or consistent effect on the complement titer within the first 3 hours after its injection, although it did produce a lymphocytopenia.

Table 1 summarizes the experimental procedures and the results. It is clear that injection of ACE consistently produced a lymphocytopenia and the injection of the ACE diluent alone did not consistently produce a drop in circulating lymphocytes. There was considerable delay in the appearance of precipitins in the hormone-treated rabbits, as judged by the delay in fixation of complement following the injection of antigen, compared to the fixation of complement after the second or third injection of antigen in the controls. Moreover, there was more antibody protein in the serums of the control than in the hormone-treated group. This difference was not indicated by the results of the antiserum dilution method, indicating the superiority of the quantitative determination.

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TABLE 2.—*Effect of ACE on hemagglutinin formation.*

Rabbit no.	Treatment	Time*	Titer									
			20	40	80	160	320	640	1280	2560	5120	10,240
S-1	None	7	++	++	++	+	+	+	+	+	+	−
S-2	None	7	++	++	++	+	+	+	+	+	+	−
S-3	None	7	++	++	++	+	+	+	+	+	+	+
S-4	None	7	++	++	++	+	+	+	+	+	+	−
S-5	ACE	7	++	++	++	+	+	+	+	+	+	−
S-6	ACE	7	++	++	++	+	+	+	+	+	+	−
S-7	ACE	7	++	++	++	+	+	+	+	+	−	−
S-8	ACE	7	++	++	++	+	+	+	+	+	+	−

Immunization schedule: daily intravenous injections of 1 ml 5% sheep cells.
Hormone: 2 ml ACE subcutaneously daily.
* Time in weeks after immunization was begun.

Effect of ACE on the production of hemagglutinins

Since Hammond and Novak[15] had also reported an enhancing effect of ACE on hemagglutinin production, the next experiment was concerned with the effect of the hormone on the antibody response of rabbits to sheep red blood cells and their antibody titers after they had fallen to zero or low values. Again the experimental details were patterned after those of Chase, Dougherty, and White.[5]

In tables 2 and 3 are summarized the experimental conditions and results. There was little effect of the hormone either on the acute antibody response or the production of an increase in hemagglutinin once it had dropped to low values. In fact, there seemed to be a drop in titer in two of the rabbits given hormone.

On the assumption that the dilutions of serum were too widely separated to detect small changes in agglutinin titer, the antibody titrations were repeated by splitting the doubling dilutions into smaller intervals. However, even by this more sensitive method little or no difference in hemagglutinins could be shown between the control and hormone-treated groups.

TABLE 3.—*Attempt to produce a nonspecific anamnestic reaction with ACE.*

Rabbit no.*	Treatment	Time in hrs.	Titer									
			20	40	80	160	320	640	1280	2560	5120	10,240
S-1	ACE	0	+	+	+	−	−	−	−	−	−	−
		3	+	+	+	−	−	−	−	−	−	−
		6	+	+	−	−	−	−	−	−	−	−
		9	+	+	−	−	−	−	−	−	−	−
		24	+	+	+	−	−	−	−	−	−	−
S-2	ACE	0	+	+	−	−	−	−	−	−	−	−
		3	+	−	−	−	−	−	−	−	−	−
		6	+	−	−	−	−	−	−	−	−	−
		9	+	−	−	−	−	−	−	−	−	−
		24	+	−	−	−	−	−	−	−	−	−
S-3	ACE	0	−	−	−	−	−	−	−	−	−	−
S-5		3	−	−	−	−	−	−	−	−	−	−
S-6		6	−	−	−	−	−	−	−	−	−	−
		9	−	−	−	−	−	−	−	−	−	−
		24	−	−	−	−	−	−	−	−	−	−

* Immunized for 7 weeks as in table 2. This experiment was done 13 weeks after immunization was begun.

Effect of ACE on the primary and secondary antitoxic response to the injection of diphtheria toxoid

Previous studies on antitoxin formation[17,18] showed that considerable amounts of diphtheria antitoxin were synthesized in the popliteal lymph node

TABLE 4.—*Effect of ACE on primary and secondary antitoxic response to diphtheria toxoid.*

Rabbit no.	Treatment	Time in days	Antitoxin units/ml serum
1	No ACE	II-8	35
3	No ACE	II-8	35
5	No ACE	II-8	20
15	ACE I	I-21	0.5
		II-8	15.0
17	ACE I	I-21	0.5
		II-8	0.5
19	ACE I	I-21	0.3
		I-8	4.0
10	ACE II	I-21	0.1
		II-8	8.0
11	ACE II	I-21	0.1
		II-8	3.0
14	ACE II	I-21	0.1
		II-8	10.0
18	ACE II	I-21	
		II-8	5.0

Immunization, each rabbit—12.5 Lf alum-precipitated diphtheria toxoid twice into left rear foot pad separated by interval of 3 weeks.

Treatment—ACE I—0.5 ml ACE and 4.5 ml ACE intravenously into left foot pad for 3 days before primary injection of antigen. ACE II—0.5 ml ACE in left foot pad and 4.5 ml ACE intravenously for 3 days before secondary injection of antigen.

Time—I-21 means 21 days after primary injection and II-8 means 8 days after the second injection.

following injection of toxoid into the corresponding foot pad. Therefore, it was possible to study the effect of the hormone on this local antitoxin formation. Moreover, the great difference between the primary and secondary antitoxic responses to diphtheria toxoid[26] permitted the study of the influence of ACE on each of these phases. Accordingly, the hormone preparation was injected before the primary injection of toxoid in one group of animals and just before the second injection of toxoid in another.

Table 4 summarizes the entire experiment. The results were very inconsistent. They varied from almost no effect on the primary or secondary response to almost complete inhibition of the secondary response. In no case, however, was there any evidence of stimulation of the primary or secondary response. There was no correlation between the magnitude of the primary and secondary responses.

Relation of ACE to the nonspecific anamnestic reaction

In the course of studies on the mechanisms of antitoxin formation[18] it was found that the injection of tetanus toxoid into rabbits previously given a mixture of diphtheria and tetanus toxoids often led to the appearance of diphtheria antitoxin in the blood several days later. Typical results comparing the nonspecific (Tet II) with the specific (Dip II) antitoxic response are shown in table 5.

Since Dougherty and White[3] had implicated ACE in the production of the anamnestic reaction an experiment was designed to determine whether these hormones could be related to the anamnestic reaction. In the first experiment the second injection of tetanus toxoid was replaced by the injection of ACE to

26. Glenny, A. T. and Südmersen, H. J. 1921, J. Hyg. **20**: 176–220.

TABLE 5.—*Anamnestic reaction in rabbits following injection of tetanus toxoid.*

Rabbit no.	Treatment	Diphtheria antitoxin units/ml					
		Date					
		2/9	3/4	3/8	3/12	3/19	3/29
149	Tet II	0.001	0.05	0.10	*1.5*	1.5	0.20
151		0.001	0.10		*1.5*	0.01	0.5
152	Dip II	0.001	0.1	*1.5*	*5.0*	3.0	
154		0.001	0.2	*1.0*	*10.0*	0.5	

Tet II—Rabbits on 2/9 injected in rear foot pad with 0.5 ml of mixture of 0.25 ml (5 Lf) alum-precipitated tetanus toxoid plus 0.25 ml (10 Lf) alum-precipitated diphtheria toxoid, followed 3 weeks later (3/4) by a similar injection of 0.25 ml of tetanus toxoid.

Dip II—Same primary injection as other group but given 0.25 ml (10 Lf) diphtheria toxoid as second injection of 2/9.

TABLE 6.—*Attempt to produce nonspecific anamnestic response by injection of ACE.*

Rabbit no.	Antitoxin units/ml						
	Date						
	5/19	6/11	6/12	6/13	6/14	6/15	6/21
1 HS	0.001	0.02	0.05	0.05	0.05		0.05
F 1	0.001	0.02	0.02		0.01	0.05	
F 2	0.001	0.02	0.02	0.02	0.02	0.02	
F 3	0.001		0.10	0.25		0.10	
F 4	0.001	0.02	0.02	0.02	0.02	0.02	

Protocol: 5/19 8 Lf alum-precipitated toxoid intravenously.
6/12 10 ml ACE subcutaneously.

TABLE 7.—*Attempt to produce nonspecific anamnestic response by injection of ACE.*

Rabbit no.	Antitoxin units/ml				
	Hours				
	0	3	6	9	24
1 HS	0.05	0.05	0.02	0.02	0.05
F 1	0.02	0.02	0.02	0.02	
F 2	0.02	0.02	0.02	0.02	0.02
F 3	0.10	0.10	0.10	0.10	0.25
F 4	0.02	0.02	0.02	0.02	0.02

Protocol: Same as in table VI, but shows antitoxin level at intervals during first 24 hours after injection of ACE.

determine whether a similar nonspecific reaction might be produced. As shown in table 6 and 7 the injection of ACE usually did not cause the appearance of even small amounts of antitoxin in the serum, compared to the amount present at the time of injection of hormone. The single exception is rabbit F3, which showed an increase 24 hours after injection. Occasionally, there was a drop in antitoxin several hours after the injection of ACE.

Following this experiment, the possibility was considered that the endogenous secretion of adrenal cortical hormones might somehow produce an anamnestic reaction, while the injection of these hormones might not. Accordingly, it was determined whether the injection of tetanus toxoid led to increased secretion of adrenal cortical steroids. In view of the ability of these hormones to cause a lymphocytopenia and a decrease in complement,[25] the blood of the rabbits was examined at various intervals after injection of the tetanus toxoid. As shown in table 8, there was no evidence of a drop in complement or a lymphocytopenia of any great magnitude following the administration of toxoid.

In the next experiment the ACE was injected into rabbits which had received two injections of diphtheria toxoid about 3 weeks apart, had experienced the secondary antitoxic response, and then had lost most of their circulating antitoxin. As in the previous experiments, there was no demonstrable effect of the hormone on the level of antitoxin.

Effect of ACE on the local formation of shigella agglutinins

As an outgrowth of studies by Ehrich and Harris[27] and by this laboratory,[18] a method was developed to limit antibody formation in the rabbit largely to the local lymph node. The minimum

27. Ehrich, W. E. and Harris, T. N. 1945, Science. **101**: 28–31.

TABLE 8.—*Complement levels and lymphocyte counts at various intervals after the injection of tetanus toxoid into rabbits previously immunized with a mixture of tetanus and diphtherial toxoids.*†*

Rabbit no.	C′ titer Hours									Lymphocytes/cu mm Hours								
	0	3	6	9	12	24	48	72	96	0	3	6	9	12	24	48	72	96
F 1	0.60	0.60	1.20	1.4	1.80	1.0	1.3	0.90	0.92	49	31	28	44	48	44	52	48	52
F 3	0.74	0.78	0.94	1.0	0.80	1.0	1.0	0.85	0.88	58	54	54	58	61	63	57	49	62
F 4	0.65	0.72	0.84	0.9	0.72	1.2	0.9	0.88	0.88	46	44	47	49	52	57	58	64	61

Lymphocytes expressed in thousands.
* Pretreatment of rabbits as in tables 6 and 7.
† All rabbits produced both diphtheria antitoxin as in table V and tetanus antitoxin as measured by the hemagglutination of toxoid-sensitized red cells.[28]

TABLE 9.—*Effect of ACE on local antibody formation.*

Rabbit no.	Date	Titer
15 L control	6/16	1:10
	6/20	1:20
	6/22	1:640
	6/24	1:2560
	6/26	1:1520
16 L control	6/16	1:10
	6/22	1:40
	6/24	1:320
	6/24	1:2560
	6/26	Dead
11 L ACE	6/16	1:10
	6/20	1:20
	6/22	1:320
	6/24	1:160
	6/26	1:80
	6/28	1:80
14 L ACE	6/16	1:10
	6/20	1:10
	6/22	1:80
	6/24	1:80
	6/26	1:10
	6/28	1:10

Controls.—6/14 one-half ml ACE diluent left foot pad. four-and-one-half ml subcutaneously same as 6/15, 6/16, 6/17, 6/18, 6/20, 6/21, 6/32, 6/24, 6/25, 6/26. On 6/17 5 μg of *Shigella* injected into foot pad 4 hours after the hormone.

ACE.—Same as controls but ACE used instead of its diluent.

number of bacteria required for the local formation of antibodies upon injection into the foot pad was first determined. Under these circumstances, if the minimum number of organisms was injected and the regional popliteal lymph node removed 40 hours later, agglutinin formation did not occur. Apparently in 40 hours most of the antigen had been carried to and fixed in the lymph node and was removed when the node was extirpated. The hormone was injected for 3 days before the injection of antigen. The antibodies were determined by the dilution technique because they were present in too low a concentration to be detected by the quantitative agglutination technique. However, as shown in table 9, the results show considerable inhibition of agglutinin formation in the hormone- as compared to the untreated rabbits.

DISCUSSION

It is difficult to understand the failure to observe enhancement of antibody formation under experimental conditions very similar to those employed by the previous authors.[5] The hormone preparation was very active, as indicated by the consistent development of a profound lymphocytopenia following its administration. Bleedings were often made during or after the loss of lymphocytes from the peripheral blood at a time when release of protein from these cells would be expected to be maximal. Measurement of antibody concentration by dilution of antiserum, as done by Chase, Dougherty, and White,[5] did not detect an enhancing effect of hormone. It is possible that in our experiments considerable endogenous secretion of adrenal cortical steroids followed the handling and bleeding of the rabbits, so that the control animals in fact experienced secretion of these steroids equivalent to that in rabbits injected with ACE. This possibility was considered likely since in previous experiments from our laboratory[25] lymphocytopenia had been observed following handling of rabbits. However, this explanation of our results does not appear valid since: (1) Increased secretion of adrenal cortical steroids would just as likely have occurred in the control rabbits as in the original experiments.[5] (2) If such secretion occurred in control animals it might be expected to produce either no effect or the actual inhibition of antibody formation which was observed in most of our experiments.

In the experiments employing diphtheria toxoid as the antigen, very sensitive and precise methods were available for the detection of small differences in antitoxin production in the control and hormone-treated animals. For instance, the rabbit intracutaneous method permits the determination of as little as 0.25 μg of antitoxin protein. It would appear, therefore, that if the injection of hormone caused any changes in the concentration of antitoxin these changes

were not very great and were certainly not comparable to those produced by the injection of tetanus toxoid into rabbits previously immunized with diphtheria toxoid. Thus these experiments have shown that there is at least one system (diphtheria toxoid-tetanus toxoid) where a nonspecific anamnestic response may be produced without any evidence of a relationship to the secretion of adrenal cortical hormones. Comparing the anamnestic reaction produced by the injection of tetanus toxoid with that following the injection of ACE as reported by Chase, Dougherty and White,[5] several differences are evident: (1) The toxoid leads to the appearance of antibody several days after injection, whereas the hormone was followed in 3 to 6 hours by the appearance of antibody which dropped by the end of 24 hours to original level. (2) No lymphocytopenia followed the injection of toxoid. (3) The ACE led to a great increase in antibody level over that originally present, whereas a comparatively much smaller increase was caused by the toxoid in the present experiments.

From the data presented it must be concluded that the enhancement of antibody production and the production of an anamnestic response by the injection of ACE do not occur regularly or require very special conditions. The more common reaction following treatment with hormone seems to be inhibition of antibody formation. To demonstrate an enhancing effect of ACE it may be necessary to immunize rabbits for a longer period of time than was done in the present study. Such hyperimmunized rabbits might then possess considerable amounts of residual antibody in the tissues. However, such "tissue antibody" was not found in any of a group of rabbits hyperimmunized with egg albumin or diphtherial toxoid. These results raise the possibility that the induction of the anamnestic reaction either by the injection of ACE[5] or tetanus toxoid does not involve merely the release of preformed antibody but more likely the de novo synthesis of antibody from suitable precursors during the period following the injection of hormone or tetanus toxoid. Further experiments are being conducted to determine the validity of the latter hypothesis.

SUMMARY

Egg albumin was injected into two groups of animals, the first receiving adrenal cortical extract, the second only the hormone diluent, with each injection of antigen. The hormone caused a delay in the appearance of precipitins and resulted in a final lower concentration of circulating antibody compared to the controls. The ACE did not appear to enhance the production of sheep hemagglutinins in rabbits and did not produce an increase of this antibody once it had dropped to low values. It was found that ACE occasionally reduced the secondary response to diphtheria toxoid but did not lead to an increase under conditions of local antitoxin formation.

It was found that the injection of tetanus toxoid into rabbits previously inoculated with a mixture of tetanus and diphtheria toxoids often resulted within several days in the appearance of diphtheria antitoxin as well as tetanus antitoxin in the serum. There was no evidence that the injection of tetanus toxoid had led to appreciable secretion of adrenal cortical hormones.

The injection of ACE into rabbits which had received two injections of diphtheria toxoid, had developed a secondary response, and then had lost this antibody did not cause an increase in the serum antitoxin.

Injection of ACE for 3 days before the administration of *Shigella* into the foot pad of the rabbit effected considerable inhibition of agglutinin formation.

Possible reasons for the inability to confirm the original observations of Dougherty and White were given. It was concluded that the production of enhanced antibody formation or the anamnestic reaction by the injection of ACE does not occur regularly in immunized rabbits or requires very special conditions. The possibility was put forth that the induction of the anamnestic reaction by either ACE or tetanus toxoid might involve the de novo synthesis of antibody protein during the period following the injection.

ENERGY REQUIREMENTS FOR SYNTHESIS OF FELINE PNEUMONITIS VIRUS IN ISOLATED CHICK EMBRYO YOLK SAC

JAMES W. MOULDER, BETTY R. S. MCCORMACK, AND MARGARET K. ITATANI
From the Department of Bacteriology and Parasitology and the Department of Biochemistry, The University of Chicago, Chicago 37, Illinois

The synthesis of virus in an infected cell requires the expenditure of considerable metabolic energy, and if, as is generally assumed, the virus has no energy-producing mechanisms, this energy must be generated by the infected cell itself. Recent reports that malonate (Ackermann, 1951a) and fluoracetate (Ackermann, 1951b; Ainslee, 1952) inhibit the multiplication of influenza and poliomyelitis viruses implicate the Krebs' cycle as a possible source of the energy for the multiplication of these viruses.* The present study deals with the energy-yielding mechanisms of chick embryo yolk sac and how they are related to the multiplication in that tissue of the agent of feline pneumonitis, a member of the psittacosis-lymphogranuloma group of viruses. The data suggest that the main source of metabolic energy in yolk sac is high energy phosphate bonds generated by the aerobic oxidation of endogenous substrates, probably fatty acids, and that the growth of the virus is dependent upon this energy source.

METHODS

Methods for studying the respiratory metabolism of chick embryo yolk sac.—The histology of the yolk sac and its suitability for manometric measurements has been fully discussed elsewhere (Moulder and Weiss, 1951a). Yolk sacs from White Leghorn chick embryos 8 to 10 days old were removed, washed and weighed as already described (Moulder and Weiss, 1951a). Portions of the yolk sac weighing 290 to 310 mg were transferred to 5 ml Warburg flasks containing 1.0 ml balanced salt solution. The basic salt solution was composed of 0.136 *M* NaCl, 0.005 *M* KCl, 0.001 *M* $MgSO_4$, 0.002 *M* KH_2PO_4, and 0.01 *M* Krebs' phosphate buffer, pH 7.4 (Krebs and Eggleston, 1940). When oxidative phosphorylation was studied, the Krebs' phosphate buffer was replaced by 0.01 *M* glycyl-glycine, pH 7.4 (glycyl-glycine is not oxidized by yolk sac). After a 10 minute equilibration, oxygen consumption and respiratory quotients were determined in air at 37 C by the direct method of Warburg (Dixon, 1943). For measurement of anaerobic glycolysis, 0.02 *M* $NaHCO_3$ replaced the phosphate buffer and the gas phase was 5% CO_2—95% N_2.

When chemical analyses were made, the entire contents of each Warburg flask were quantitatively transferred to an all-glass Potter-Elvehjem homogenizer and homogenized in the cold. For glucose determinations, the sample was homogenized in the $ZnSO_4$—$Ba(OH)_2$ mixture of Nelson (1944); 5% trichloracetic acid was used for all other purposes. The homogenate was centrifuged at 0 C and the supernatant was filtered and kept at 0 C until analyzed. Initial values for all determinations were obtained by homogenizing samples of yolk sac immediately after they were removed from the embryo. The following analytical procedures were employed: inorganic phosphate (Ernster, Zetterstrom and Lindberg, 1950), total phosphorus (Zahler, 1953), glucose (Nelson, 1944), lactate (Barker and Summerson, 1941) and pyruvate (Friedemann and Haugen, 1943; Speck, Moulder and Evans, 1946).

Radioactive phosphorus 32 was obtained from the Oak Ridge National Laboratories. The radiochemical techniques employed have been described by Zahler (1953).

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* After this paper was submitted for publication, Ackermann and Johnson (1953) reported that 2,4-dinitrophenol inhibits the growth of influenza virus in surviving chorioallantoic membranes and concluded that the energy for virus synthesis is derived from oxidative phosphorylations carried out by the host cell.

Methods for studying virus growth in isolated yolk sac.—The characteristics of feline pneumonitis virus and procedures for inoculating chick embryos with virus, for harvesting infected yolk sacs and for titrating virus infectivity in terms of chick embryo LD_{50}'s have been given in detail in a previous report (Moulder and Weiss, 1951b).

Six-day-old chick embryos were inoculated via the yolk sac with about 10^4 LD_{50}'s of feline pneumonitis virus, an inoculum sufficient to kill half of the embryos by the end of the fourth day after infection. On the third day after virus inoculation, the yolk sacs were removed from the embryos, and without any washing, one-fourth of each yolk sac, about 200 mg wet weight, was removed and incubated in vitro. The remaining portions of at least 4 yolk sacs were pooled and emulsified by shaking with glass beads on a mechanical shaker with 2 times their weight of Krebs' phosphate buffer for 15 minutes at room temperature. The emulsion was then frozen at −70 C until used for determination of the initial virus titer.

Each one-fourth of a yolk sac was placed in 5 ml of bicarbonate-saline contained in a 25 ml Erlenmeyer flask with side arms for passing a gas mixture through it. The composition of the bicarbonate-saline was: 0.025 *M* $NaHCO_3$, 0.120 *M* NaCl, 0.005 *M* KCl, 0.003 *M* $CaCl_2$, 0.001 *M* $MgSO_4$, and 0.001 *M* KH_2PO_4. With 5% CO_2, the initial pH was about 7.5. When other electrolytes, such as NaF were added, the NaCl concentration was appropriately reduced. The flasks were placed at 37 C and gently rocked while a mixture of 95% air and 5% CO_2 was passed through the side arms. After 24 hours at 37 C, 1 ml Krebs' phosphate buffer was added to each flask and the entire contents were emulsified and kept at −70 C until determination of the final in vitro virus titer.

On the fourth day after virus inoculation, at the same time the final in vitro samples were taken, the yolk sacs of at least 4 living embryos which had been infected at the same time as the others were removed and used to prepare an emulsion for estimating the final in vivo virus titer.

Thus, from the initial virus titer and from the final in vitro and in vivo virus titers, it was possible to calculate the extent of virus multiplication in the isolated yolk sacs and to compare this virus growth with that occurring in the yolk sacs of intact embryos during the same interval.

All operations were performed aseptically and each virus sample titrated was bacteriologically sterile.

RESULTS

Energy-yielding mechanisms in chick embryo yolk sac

Since it had already been established that infection with feline pneumonitis virus does not alter the rate of oxygen consumption or anaerobic glycolysis in yolk sac (Moulder and Weiss, 1951a), it was considered sufficient to use only uninfected yolk sacs in the short-term experiments on energy metabolism.

Endogenous respiration.—Yolk sac consumes oxygen for many hours in the absence of any added substrate (fig. 1). Oxygen uptake is almost constant for 2 hours and then begins to slacken, but at the end of 4 hours oxygen is still used at 65% of the initial rate. In 7 determinations, the mean respiratory quotient was 0.69. This value, which is almost theoretical for the complete oxidation of long-chain fatty acids, together with the very high lipid content of yolk sac (Moulder and Weiss, 1951a; Zahler, 1953), suggests that the endogenous substrates are fatty acids. No detectable amounts of acetate or acetoacetate were formed during oxidation of the endogenous substrates. If long-chain fatty acids are indeed the substrates for the endogenous oxygen uptake, they must be oxidized completely to CO_2 and H_2O.

The effect of some common metabolic inhibitors on the endogenous respiration of yolk sac is shown in figure 1. In the presence of 0.001 *M*, NaN_3 or NaF, oxygen uptake is strongly inhibited and almost ceases within 4 hours. However, the inhibition during the first hour of observation is only moderate. Both malonate and monofluoracetate exert a small but definite inhibition in 0.01 to 0.02 *M* concentrations. They do not inhibit the respiration of whole sucrose homogenates to any greater degree, so their relatively small inhibitory actions

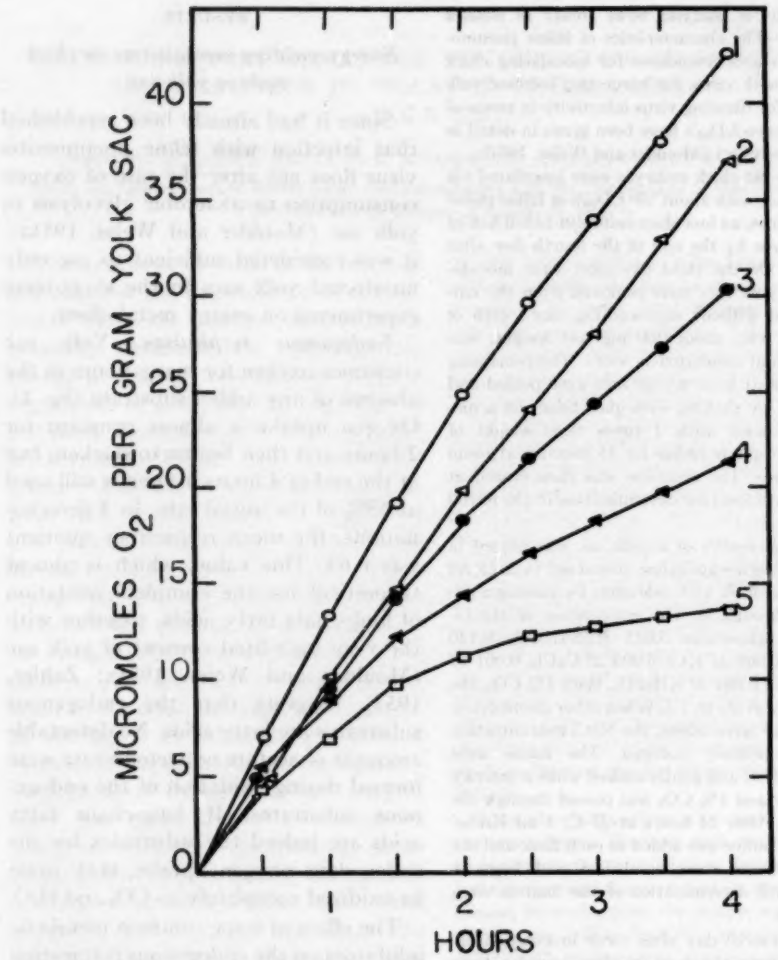

FIG. 1.—Effect of inhibitors on the endogenous respiration of chick embryo yolk sac. (1) no inhibitor added. (2) 0.01 *M* sodium monofluoroacetate. (3) 0.01 *M* sodium malonate. (4) 0.01 *M* NaF. (5) 0.001 *M* NaN_3.

cannot be explained by failure to penetrate the intact yolk sac cells. In concentrations as high as 0.0002 *M*, 2,4-dinitrophenol does not lessen the oxygen uptake of yolk sac. In general, oxidation of the endogenous substrates of yolk sac is comparatively resistant to a variety of metabolic inhibitors; the presence of a high concentration of inhibitor over a period of several hours is required for any marked effect.

Metabolism of pyruvate.—Yolk sac rapidly utilizes pyruvate aerobically (table 1). Oxygen consumption increases and the respiratory quotient rises. The inhibitors shown in figure 1 have about the same effect on the oxidation of pyruvate as on the oxidation of the en-

TABLE 1.—*Carbohydrate metabolism in chick embryo yolk sac.*

Substrate added	Measurement	Micromoles per gram yolk sac			
		No added substrate		0.01 *M* added substrate	
		Aerobic	Anaerobic	Aerobic	Anaerobic
Pyruvate	Pyruvate used			18.6	6.1
	Oxygen used	25.9		38.1	
	Respiratory quotient	0.69		0.97	
Glucose	Glucose formed	20.6	3.0	17.3	6.7
	Lactate formed	1.7	12.3	4.0	16.7
	Glucose formed plus ½ lactate formed	21.5	9.2	19.3	15.1
	Oxygen used	24.9		21.4	
	Respiratory quotient	0.70		0.68	
	Acid formed in bicarbonate		12.3		17.6
	Acid formed in bicarbonate / Lactate formed		1.00		1.05

The incubation period was 2 hours.

dogenous substrates. There is a slow anaerobic removal of pyruvate.

Metabolism of glucose.—Yolk sac exhibits an unusual glucose metabolism. There does not seem to be any aerobic glucose oxidation at all (table 1). The addition of glucose alters neither the oxygen uptake nor the respiratory quotient of yolk sac. In both the presence and absence of added glucose, there accumulate considerable quantities of a yeast-fermentable reducing substance which is probably glucose. The rate of aerobic lactate formation is comparatively low.

Under anaerobic conditions, the production of lactate increases several-fold. All of the CO_2 liberated from bicarbonate buffer can be accounted for as lactate. Glucose also accumulates anaerobically, and the sum of the glucose plus one-half the lactate formed is approximately equal aerobically and anaerobically. The glucose is probably produced by the hydrolysis of yolk sac glycogen (Zwilling, 1951).

The behavior of yolk sac toward glucose and pyruvate involves an apparent inconsistency. Glucose is glycolyzed to lactate, undoubtedly through pyruvate, and yet pyruvate is readily oxidized while glucose is not. The inability of the yolk sac to oxidize its carbohydrate stores is further evidence for believing that the endogenous substrates are fatty acids.

Oxidative phosphorylation.—Oxidative phosphorylation in yolk sac was studied in terms of changes in the inorganic phosphate level and of the esterification of inorganic orthophosphate labeled with P^{32} into the trichloracetic acid soluble organic phosphate compounds. Inorganic orthophosphate was separated from trichloracetic acid filtrates as the phosphomolybdate complex by the isobutanol-benzene extraction procedure of Ernster et al (1950) and directly determined in the extract as described by these authors, while the acid-soluble organic P^{32} was counted in the aqueous residue remaining after extraction of the inorganic phosphate. Although the uptake of inorganic phosphate in yolk sac was small, necessitating the use of low phosphate concentrations, the separation of inorganic phosphate was very efficient and quite reproducible results were obtained.

When yolk sac is incubated in glycylglycine-saline alone, there occurs a rapid release of about 5 micromoles of inorganic phosphate per gram of yolk sac, probably from the hydrolysis of ATP, since yolk sac has a high ATP-ase activity. Only a barely detectable amount of P^{32} is esterified (table 2). When 0.02 *M* NaF is added there is a small but

TABLE 2.—*Oxidative phosphorylation in chick embryo yolk sac.*

	Length of exp. in minutes	Oxygen used, micromoles per gram yolk sac	Change in inorganic P, micromoles per gram yolk sac	$P^{32}O_4^{=}$ esterified, percent of total added
None	30	11.5	+4.7	2.4
Glucose	30	9.8	+2.9	1.5
NaF	30	5.5	−1.3	7.3
Glucose, NaF	30	7.1	−1.9	9.3
Glucose, NaF, 2,4-dinitrophenol	30	6.5	+3.3	3.1
Glucose, NaF, NaN_3	30	5.4	−1.1	6.7
None	60	17.3	+6.3	1.9
Glucose, NaF	60	11.6	−3.0	11.3
Glucose, NaF,	60	11.1	+2.7	3.1

The results at 30 and 60 minutes were obtained on different days. The concentrations of the added substances were: 0.01 *M* glucose, 0.02 *M* NaF, 0.0002 *M* 2,4-dinitrophenol and 0.001 *M* NaN_3. The reaction mixture contained 35,000 counts per minute per ml P^{32}. The counts on the various samples were from 2 to 6 times background.

consistent decrease in inorganic phosphate and considerably more P^{32} is esterified. It may reasonably be assumed that NaF promotes phosphorylation by inhibiting the breakdown of ATP. The addition of glucose alone is without marked effect, but maximum phosphorylation is obtained when both glucose and NaF are added. Since glucose is not oxidized, it probably functions only as a phosphate acceptor. Other substances, such as adenine nucleotides, dicarboxylic acids, DPN, etc., have no effect on phosphorylation. Even in the presence of glucose and NaF, the phosphorylating system of yolk sac deteriorates rapidly. As seen from table 2, there is about as much phosphorylation in 30 minutes as in 60.

Although the rate of oxidative phosphorylation in yolk sac is small in comparison with certain other well-known systems, such as liver mitochondria, is it sufficient to produce a rapid turnover in the acid-soluble phosphorus fraction of that tissue. It may be estimated from the data of table 2 that the initial rate of inorganic phosphate uptake in isolated yolk sac is at least 3 to 4 micromoles per gram yolk sac per hour. Therefore, the 5 micromoles labile phosphorus per gram, probably ATP, would come into equilibrium with the inorganic phosphorus in less than 2 hours, while the entire 35 micromoles acid-soluble phosphorus per gram would reach equilibrium in 12 hours. In the intact embryo, the rate of phosphorylation is probably even greater.

2,4-Dinitrophenol effectively decouples oxidation and phosphorylation at 0.0001 to 0.0002 *M* concentrations but has no effect at 0.00001 *M* (table 2). Sodium azide is not a powerful decoupling agent in yolk sac, even in concentrations at which it significantly inhibits oxygen uptake (table 2).

Energy requirements for multiplication of feline pneumonitis virus in isolated yolk sac

The results of the preceding section strongly suggest that the energy requirements of chick embryo yolk sac are met by the aerobic oxidation of endogenous substrates, probably fatty acids, with the concomitant formation of high energy phosphate bonds through the coupling of oxidation and phosphorylation. Here, the relation of these energy-yielding mechanisms to the multiplication of feline pneumonitis virus is considered. For this purpose, fragments of yolk sac from chick embryos already infected with the virus were incubated in bicarbonate-saline and the effect of variations in environmental conditions on virus multiplication was noted. Such a technique is certainly not the best for maximum virus multiplication in vitro, but it is one of the simplest and serves to define the minimum requirements for significant virus reproduction. The general rationale of the method can perhaps best be understood by considering it the study of virus reproduction in a tissue slice.

Multiplication of the virus in isolated

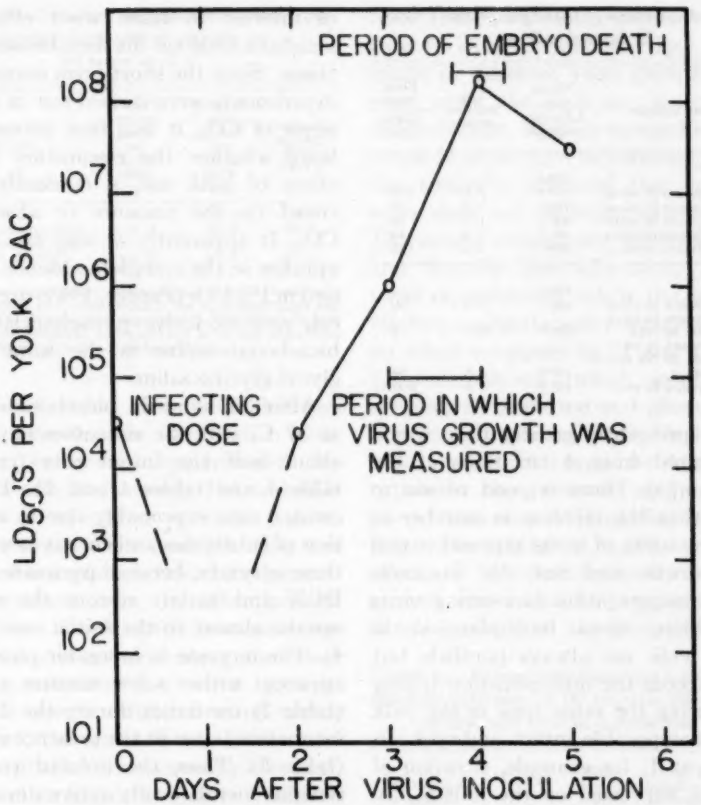

FIG. 2.—The growth curve of feline pneumonitis virus in chick embryo yolk sac. The values for 3 and 4 days are the means obtained from 20 experiments on the growth of the virus in isolated yolk sac. At 1 day, the virus titer was less than 10^8 LD_{50} per yolk sac and could not be precisely estimated.

yolk sac.—A brief description of the growth curve of feline pneumonitis virus in the yolk sacs of intact chick embryos should explain the time sequence chosen for study of virus growth in isolated yolk sac (fig. 2). After a sharp drop in virus titer following inoculation, the number of LD_{50}'s in the yolk sac increases rapidly until the embryo dies and then slowly declines (Moulder and Weiss, 1951a). The growth curve is highly reproducible and almost all infected embryos die within a few hours of each other. The near logarithmic increase in virus is such that 90 to 99% of all virus multiplication, both in intact embryos and in isolated yolk sacs, occurs from the third to the fourth day after virus inoculation,—the period of observation in these experiments (fig. 2).

In experiment 1 of table 3, fragments of yolk sac were removed from 4 different embryos 3 days after they were infected with virus and incubated aerobically in bicarbonate-saline for 24 hours. After incubation, each yolk sac fragment contained 40 to 100 times as many LD_{50}'s of virus as a comparable pooled sample titrated at the beginning of the incubation period. This variation

TABLE 3.—*Growth of feline pneumonitis virus in isolated yolk sac.*

Yolk sac sample	Virus titer, LD_{50} per yolk sac	Log increase in titer	Final titer / Initial titer
Experiment 1			
Initial	$10^{5.0}$		
After 24 hours in vitro	$10^{7.0}$	2.0	100
After 24 hours in vitro	$10^{7.9}$	1.9	79
After 24 hours in vitro	$10^{7.7}$	1.7	50
After 24 hours in vitro	$10^{7.6}$	1.6	40
After 24 hours in vivo	$10^{8.1}$	2.1	126
Experiment 2			
Initial	$10^{6.5}$		
After 24 hours in vitro	$10^{7.4}$	1.9	80
After 24 hours in vitro with 0.01 *M* NaF	$10^{6.6}$	0.1	1
After 24 hours in vitro with 0.01 *M* Na fluoracetate	$10^{7.1}$	1.6	40
After 24 hours in vivo	$10^{7.8}$	2.3	200

is not significantly greater than would be expected from 4 titrations of the same sample. There is good reason to believe that the increase in number of infectious units of virus represents real virus growth and not, for instance, merely disaggregation of existing virus units. First, virus multiplication in isolated yolk sac always parallels but never exceeds the multiplication taking place during the same time in the yolk sacs of comparable intact embryos. In experiment 1, for example, titration of yolk sacs harvested on the fourth day of infection showed that the virus multiplied 126-fold in the yolk sacs of intact embryos during the period of in vitro incubation (table 3). Second, examination of histological sections of infected yolk sac before and after incubation in bicarbonate-saline clearly shows a great increase in the actual mass of virus. Third, penicillin G, a known inhibitor of feline pneumonitis virus multiplication (Weiss, 1950), completely prevents the increase in virus titer in concentrations as low as 0.1 unit per ml.

Effect of the 24-hour incubation on the metabolism of yolk sac.—After it was established that significant multiplication of feline pneumonitis virus occurs in isolated yolk sac incubated aerobically in bicarbonate-saline for 24 hours, it was of interest to learn what effect this incubation has on the metabolism of the tissue. Since the short-term manometric experiments were carried out in the absence of CO_2, it was first necessary to learn whether the respiratory metabolism of yolk sac is markedly influenced by the presence or absence of CO_2. It apparently is not; the oxygen uptakes in the complete absence of CO_2 and in 1% CO_2 (Pardee, 1949) are identical, and oxidative phosphorylation in bicarbonate-saline is the same as in glycyl-glycine-saline.

After 24 hours in bicarbonate-saline at 37 C, yolk sac consumes oxygen at about half the initial rate (compare table 4 and tables 1 and 2). The decreased rate is probably due to exhaustion of substrates and coenzymes rather than enzymes, because pyruvate, ATP, DPN and malate restore the oxygen uptake almost to the initial rate (table 4). The increase in inorganic phosphate apparent within a few minutes at 37 C (table 2) continues during the 24-hour incubation, even in the presence of NaF (table 5). Thus, the isolated yolk sac remains metabolically active during the period of virus growth but slowly loses its organic phosphorus compounds, coenzymes, and oxidizable substrates.

Effect of environmental conditions and metabolic inhibitors on virus multiplication.—Changing the conditions of incu-

TABLE 4.—*Respiration of isolated chick embryo yolk sac after 24 hours at 37 C.*

Additions after 24 hours at 37 C	Oxygen used, micromoles per gram yolk sac
None	5.3
ATP, DPN, malate	9.9
Pyruvate	7.9
Pyruvate, ATP, DPN, malate	12.0

Yolk sacs from 9-day uninoculated embryos were kept for 24 hours at 37 C under the exact conditions used for the in vitro virus growth studies. The yolk sacs were then removed to phosphate-saline, additions were made as shown below, and respiration was measured as usual for 60 minutes. The concentrations of the added substances were: 0.01 *M* Na pyruvate, 0.001 *M* ATP, 0.0001 *M* DPN and 0.0005 *M* Na L-malate.

TABLE 5.—*Release of inorganic phosphate from isolated chick embryo yolk sac in 24 hours at 37 C.*

Sample	Total P, micromoles per gram yolk sac	Inorganic P, micromoles per gram yolk sac	Inorganic percent P, as total P
Initial	98.0	5.6	5.7
After 24 hours in vitro	118.2	36.0	30.4
After 24 hours in vitro with 0.01 *M* NaF	113.8	37.6	33.1

Yolk sacs from 9-day uninoculated embryos were kept for 24 hours at 37 C under exactly the same conditions employed in the virus growth experiments. Trichloracetic acid filtrates were prepared and analyzed as in the short-term experiments on oxidative phosphorylation.

bation and adding metabolic inhibitors yielded considerable information concerning the energy requirements for virus growth. Experiment 2 of table 3 is a typical experiment of this nature. Since as much virus multiplication was obtained in a plain aerobic bicarbonate-saline medium as in any other, growth under these simple conditions was taken as a standard for evaluating the effect of any treatment on virus growth. Fluoride clearly inhibited virus multiplication; the virus titers before and after incubation were virtually identical. Fluoracetate appears to have inhibited virus growth by 50%. However, this much variation was encountered among the identical samples of experiment 1, and the inhibition produced by fluoracetate was not considered significant, a conclusion supported by other experiments. This experiment furnishes a good example of the limitations of the technique. To show that a factor definitely prevents virus multiplication, it must be tested in such a way as to give almost 100% inhibition. This limitation is imposed chiefly by the inaccuracy of virus titrations in chick embryos, which at best indicate only 2-fold variations in titer.

Table 6 summarizes many experiments on the effect of a variety of factors on feline pneumonitis virus multiplication. Addition of either glucose or pyruvate to isolated yolk sac does not increase or decrease virus growth. However, when the yolk sac is incubated anaerobically, there is no significant increase in virus titer. Ackermann (1951a) has similarly observed that influenza virus does not multiply anaerobically. The strong respiratory inhibitors azide and fluoride also effectively prevent virus reproduction, while the weak inhibitors malonate and fluoracetate have no effect (compare fig. 1 and table 6). 2,4-dinitrophenol, which inhibits the coupling of oxidation and phosphorylation in yolk sac (table 2), also inhibits virus growth. In 6 experiments multiplication in the presence of 2,4-dinitrophenol was only 33% of that in its absence. The statistical significance of the effect of 2,4-dinitrophenol on virus growth was estimated by use of Student's *t* test, and the probability that

TABLE 6.—*Effect of environmental conditions and metabolic inhibitors on the multiplication of feline pneumonitis virus in isolated yolk sac.*

Substance added	Concentration, *M*	Number of experiments	Log increase in virus titer	Percent of standard virus growth
None (standard conditions)	0.01	22	1.49	100
Glucose	0.01	4	1.18	83
Pyruvate	0.01	3	1.40	140
Anaerobiosis		6	0.13	3
NaF	0.01	3	0.20	1
NaN_3	0.001	3	0.16	3
Malonate	0.01	3	1.53	133
Fluoracetate	0.01	3	1.42	83
2,4-Dinitrophenol	0.0001	6	0.58	33

Anaerobic conditions were produced by using 95% N_2 –5% CO_2 as the gas phase.

virus growth in the presence and absence of the inhibitor represented a single homogenous population was 0.02.

All these results are consonent with the assumption that the energy for the reproduction of feline pneumonitis virus in yolk sac is derived from high-energy phosphate bonds generated by the aerobic oxidation of the endogenous yolk sac substrates.

DISCUSSION

The respiratory metabolism of chick embryo yolk sac fits the general pattern followed by most animal tissues in its general characteristics but deviates from it in some particulars. It is unusual for the oxidation of cell lipids coupled with the esterification of inorganic phosphate to be the main pathway of energy metabolism. However, Lardy and Phillips (1941 and 1945) demonstrated a similar situation in bull spermatozoa, and it may be significant that both yolk sac and spermatozoa contain large amounts of lipid. Even more unusual is the ability of yolk sac to accumulate high concentrations of free glucose without being able to oxidize it, despite the presence of enzymes for splitting glucose to lactate and for oxidizing pyruvate. If one of the physiological functions of the yolk sac is to furnish the growing chick embryo with a steady supply of glucose (see Zwilling, 1951), then the presence of a mechanism for suppressing the oxidation of glucose within the yolk sac cell itself might serve a useful function in embryonic development.

When a chick embryo is killed by infection with feline pneumonitis virus, multiplication of the virus in the yolk sac ceases (Moulder and Weiss, 1951a). At the same time, the oxygen consumption of the tissue falls abruptly (Moulder and Weiss, 1951a), and its organic phosphorus compounds are rapidly broken down (Zahler, 1953). It cannot now be decided whether death of the embryo leads to these metabolic changes in the yolk sac or whether the biochemical derangements in the yolk sac kill the embryo. However, the close chronological correlation between cessation of virus growth and decrease in oxygen uptake and concentration of organic phosphorus compounds is in accord with the experiments reported here, which suggest that energy derived from oxidative phosphorylations is required for reproduction of the virus. Such a concept is in harmony with the assumption that the normal enzyme systems of the infected cell are diverted to the synthesis of virus.

However, not all of the enzymes of the host cell are equally vital for virus growth and some may not be necessary at all. The present experiments show that feline pneumonitis virus multiplies in yolk sac fragments which are slowly declining in oxygen uptake and in organic phosphorus. Pearson and Winzler (1949) have similarly observed that mouse encephalomyelitis virus grows well in mouse brain minces of impaired oxidative and glycolytic ability. Future investigations should more clearly reveal just which biochemical functions of the host cell are required for the growth of a given virus and which are not. It may be expected that the obligate biochemical contribution of the host will vary from virus to virus and that knowledge of this contribution will aid in the ultimate understanding of the mechanism of virus reproduction.

SUMMARY

1. Chick embryo yolk sac has a large store of endogenous substrates, probably fatty acids, which it oxidizes at an almost undiminished rate for many hours. Azide and fluoride strongly inhibit the endogenous respiration, while malonate and monofluoracetate inhibit weakly.

2. Yolk sac forms large amounts of a yeast-fermentable reducing substance, probably glucose, both aerobically and anaerobically. Yolk sac glycolyzes glucose to lactate and oxidizes pyruvate but is unable to oxidize glucose.

3. Oxidation of the endogenous substrates is accompanied by the esterifi-

cation of inorganic phosphate, which is enhanced by fluoride and glucose and is inhibited by 2,4-dinitrophenol.

4. Feline pneumonitis virus multiplies in vitro in fragments of previously infected yolk sac at a rate approaching that observed in the intact embryo, although the oxygen uptake and organic phosphorus content of the isolated tissue decrease during the period of virus growth.

5. Virus multiplication is inhibited by anaerobiosis, azide, fluoride, and 2,4-dinitrophenol, but not by malonate and fluoracetate.

6. The energy for the synthesis of feline pneumonitis virus is probably supplied by high-energy phosphate bonds generated by the aerobic oxidation of the endogenous substrates.

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THE EFFECTS OF FELINE PNEUMONITIS VIRUS INFECTION ON THE PHOSPHORUS METABOLISM OF CHICK EMBRYO YOLK SAC

STANLEY A. ZAHLER*

From the Department of Bacteriology and Parasitology, The University of Chicago, Chicago 37, Illinois

The available evidence suggests that the enzyme systems responsible for the formation of virus are present in the host cell before infection occurs. The role of the infecting virus seems to be one of regulation, directing the host's synthetic reactions towards the production of virus components rather than normal host components. Metabolic changes brought about by virus infections, then, should reflect the shifts in synthesis which accompany them. The distribution and incorporation of phosphorus in various chemical fractions of normal and infected tissues is of particular interest in such studies, because of the importance of phosphorus in the energetics of synthetic reactions as well as its presence in such important cellular compounds as the nucleic acids.

Studies on the effects of virus infections on host metabolism have been made on several host-virus systems. The work on systems consisting of *Escherichia coli* as the host and various bacteriophages containing significant quantities of desoxypentose nucleic acid (DNA) as infecting viruses has been reviewed recently.[1,2] When a phage infects a bacterial cell the antigenic proteins do not enter the cell, but remain loosely attached to its surface.[3] The DNA and perhaps other compounds enter the cell and direct the bacterial synthetic apparatus to the production of virus protein and DNA. Virus DNA appears prior to the appearance of intracellular whole phage, and almost all of the protein and DNA produced after the infection starts can be found in the progeny virus. Among the animal viruses, de Burgh et al[4] found that infection with ectromelia causes mouse livers to incorporate P into their mitochondrial and microsomal fractions at increased rates, but they found no changes in the rate of P incorporation into nuclear nucleoprotein, or in oxygen consumption, lactic acid production, or acid phosphatase activity, except for a decrease in the latter three functions during the terminal stages of infection. Gollan et al[5] found that the MM strain of encephalomyelitis virus causes a decrease in the concentration of pentose

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* Mr. and Mrs. Frank G. Logan Fellow, 1951. Present address: Department of Bacteriology, University of Illinois, Urbana, Illinois.

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nucleic acid (PNA) in infected mouse brains. In vitro studies of the growth of Theiler's GD VII polioencephalomyelitis virus in minced mouse brain by Rafelson, Winzler, and Pearson[6-8] show that the infection causes an increase in the rates of P incorporation into phospholipid and PNA, but does not affect oxygen consumption, glucose utilization, or lactic acid production. The infected tissue contains more PNA and less DNA than does normal tissue.

In the present work chick embryo yolk sacs infected with feline pneumonitis virus, a member of the psittacosis-lymphogranuloma group of viruses, were analyzed for various P-containing fractions, and the rates of P incorporation into these fractions were studied with radioactive orthophosphate. The infection causes no detectable change in the quantities of the various P-containing fractions, but it significantly increases the rate of incorporation of P into DNA, and perhaps into PNA as well.

MATERIALS AND METHODS

Yolk sacs.—A complete description of the virus, eggs, and methods of inoculating, harvesting, and titrating is given by Moulder and Weiss.[9] Eggs, or yolk sacs harvested from them, which were incubated for 10 days at 37 C were called *normal. Autolyzed* eggs were incubated for 10 days at 37 C, placed in a freezing compartment at −20 C for 6 hours to kill the embryos, and reincubated for 18 hours at 37 C to permit autolysis. Infected eggs were produced by incubating eggs for 7 days at 37 C, inoculating the living eggs with $10^{6.7}$ LD_{50}'s of feline pneumonitis virus from an emulsion of pooled infected yolk sacs diluted in veal infusion broth, and reincubating the eggs at 37 C to permit virus growth. Inoculations were made into the yolk. Almost all of the embryos died between 69 and 75 hours after virus inoculation, and about one-half of them were dead 72 hours after the inoculation. Eggs whose embryos were still alive at 72 hours were called *moribund infected* eggs (fig. 1). Eggs which had been incubated for 78 hours after inoculation, whose embryos were all dead, were called *6-hour dead infected* eggs, since their embryos had been dead an average of 6 hours. Eggs which had been incubated for 96 hours after inoculation were called *24-hour dead infected* eggs. For each sample at least 4 yolk sacs were harvested. They were immediately chilled, washed free of adsorbed yolk and albumen, and blotted dry. They were then pooled, weighed, and homogenized in a 15-ml Potter-Elvehjem homogenizer in cold 10% trichloracetic acid. Aliquots of the homogenate, usually containing 0.167 g of wet yolk sac in 1 ml of suspension, were taken for fractionation and analysis.

Radioactive phosphorus.—Radioactive phosphorus (P^{32}) was procured from the Oak Ridge National Laboratory as a weakly acidic phosphate solution. This was hydrolyzed at 95 to 100 C for 1 hour in 1 *N* hydrochloric acid to assure the predominance of orthophosphate. Concentrated sodium hydroxide was added until the solution was only faintly acidic. In the experiments using labeled phosphorus, the phosphate solution was diluted in veal infusion broth to give 134 microcuries per ml and autoclaved. Each egg was inoculated into the yolk with 0.25 ml, or 33.5 microcuries of P^{32}. The inoculation was always made on the ninth day of egg incubation. The time relationships among P^{32} inoculation, virus growth, and time of yolk sac harvest will be clarified by an examination of figure 1. It will be noted that in the tracer experiments more than 99% of the virus in infected yolk sacs was produced in the presence of P^{32}.

*Fractionation and analysis.**—The fractionation method was adapted from that of Kozloff and Putnam.[10] Each fractionation and analysis

6. Pearson, H. E. and Winzler, R. J. 1949, Oxidative and glycolytic metabolism of minced day-old mouse brain in relation to propagation of Theiler's GD VII virus. J. Biol. Chem. **181:** 577–582.
7. Rafelson, M. E., Jr., Pearson, H. E. and Winzler, R. J. 1951, In vitro inhibition of radiophosphate uptake and growth of a neurotropic virus by 5-chlorouridine. Proc. Soc. Exper. Biol. & Med. **76:** 689–692.
8. Rafelson, M. E., Jr., Winzler, R. J. and Pearson, H. E. 1949, The effects of Theiler's GD VII virus on P^{32} uptake by minced one day-old mouse brain. J. Biol. Chem. **181:** 583–593.
9. Moulder, J. W. and Weiss, E. 1951, Purification and properties of the agent of feline pneumonitis. J. Infect. Dis. **88:** 56–67.

* A detailed description of the fractionation and analytical procedures will be furnished by the author upon request.

10. Kozloff, L. M. and Putnam, F. W. 1949,

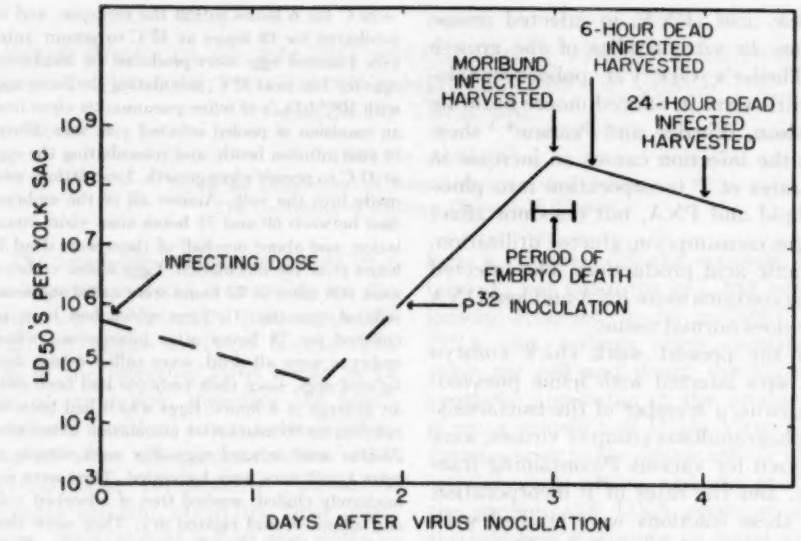

FIG. 1.—Schematic growth curve of feline pneumonitis virus in the chick embryo yolk sac.

was done in duplicate. For each single fractionation, two aliquots of the starting material (0.167 g of homogenized yolk sac in 10% trichloracetic acid) were washed several times with cold 5% trichloracetic acid to give the *acid soluble* fractions, containing inorganic phosphate and low-molecular weight organic phosphate esters. *Inorganic phosphate* in the acid soluble fraction was determined occasionally.[11–13] The method of Ernster et al[13] gave the most consistent results. The residues from the cold acid extractions were extracted with 95% ethanol, hot 3:1 ethanol-ether, and warm ether to give the *alcohol-ether soluble* fractions, containing phospholipids and similar compounds. The residues were dried in vacuo and weighed. One of the dry residues was subjected to a modification of the Schneider separation,[14] in which hot trichloracetic acid is used to extract the nucleic acids from the *phosphoprotein*. The other dry residue was subjected to a modification of the Schmidt-Thannhauser separation;[15] it was dissolved in warm potassium hydroxide, and *DNA* was precipitated by acidification. The supernatant contained PNA+phosphoprotein.

The P content of each fraction, except the acid-soluble inorganic phosphate, was determined by a modification of the Fiske-Subbarow method.[16] Radioactivity assays were made by counting liquid samples at fixed geometry with a Nuclear Instrument and Chemical Corporation D52 De-

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13. Ernster, L., Zetterstrom, R. and Lindberg, O. 1950, A method for the determination of tracer phosphate in biological material Acta Chem. Scand. **4:** 942–947
14. Schneider, W. W. 1945, Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nuclei acid. J. Biol. Chem. **161:** 293–303.
15. Schmidt, G. and Thannhauser, S. J. 1945, A method for the determination of desoxyribonucleic acid, ribonucleic acid, and phosphoprotein in animal tissues. J. Biol. Chem. **161:** 83–89.
16. Fiske, C. H. and Subbarow, Y. 1925, The colorimetric determination of phosphorus. J. Biol. Chem. **66:** 375–386.

tector Tube and Model 163 or 166 Scaling Unit. When radioactivity determinations were to be made on solid precipitates or residues, the material was first digested in sulfuric acid, and aliquots of the digest were counted in the liquid state. Values for *PNA* (grams of P, and counts /minute) were calculated as the difference between the values for nucleic acids and for DNA, or as the difference between the values for PNA+phosphoprotein, and for phosphoprotein alone. The final results were values for P content and counts/minute for acid soluble, alcohol-ether soluble, phosphoprotein, PNA, and DNA fractions, and occasionally inorganic phosphate. From these data the specific activities of the various fractions (counts/minute/gram of P) were calculated.

EXPERIMENTAL

The distribution of phosphorus in normal 10-day yolk sacs is given in table 1. The tissue contains large quantities of phospholipid and phosphoprotein P; the former may be part of the endogenous lipid energy source.[17] The PNA P:DNA P ratio is 3.5.

TABLE 1.—*The distribution of phosphorus in normal 10-day chick embryo yolk sacs.*

Fraction	Percent of total P	Mg P per 100 g wet yolk sac	Mg P per 100g dry extracted yolk sac*
Total P	100	228.4 ± 20.2†	5808
Acid-soluble P	36.7	83.7 ± 9.9	2120
Alcohol-ether soluble P	37.3	84.9 ± 8.7	2180
Phosphoprotein P	12.6	29.0 ± 3.3	743
PNA P	10.0	23.0 ± 4.4	601
DNA P	2.9	6.5 ± 0.6	164

* Weight after acid and alcohol-ether extractions.
† Mean ± standard error for 4 analyses, each in duplicate.

To determine if infection with feline pneumonitis virus changes the distribution of P in 10-day yolk sacs, analyses of moribund infected yolk sacs were compared with the values for normal tissue. These data (fig. 2) are reported in terms of multiples of the amount of DNA P present in each fraction. This value was chosen as the basis for the calculations because it has often been suggested that the DNA content of each diploid cell from any tissue of a species is constant.[18] At least in the normal tissues, the P contents in figure 2 are directly proportional to the average P content of the fraction per cell. When normal values (means of 4 analyses, each in duplicate) are compared to values for moribund infected yolk sacs (means of 6 analyses, each in duplicate) by means of Student's *t* test, the probabilities are as follows: acid soluble P, 0.93; alcohol-ether soluble P, 0.63; phosphoprotein P, 0.93; PNA P, 0.57. Thus, there are no statistically significant differences between normal and moribund infected tissues which can be demonstrated by this technique. Furthermore, no significant changes are found if the P contents are calculated as percentages of either wet or dry extracted yolk sac weight.

Although no changes in the distribution of P could be found, it was believed that changes in the rates of incorporation of P into the various fractions may be caused by the infection. Studies comparing the uptake of radioactive phosphorus in normal and infected yolk sacs were inaugurated to see if such changes are demonstrable. Radioactive P^{32}, as orthophosphate, was inoculated into the yolk of infected eggs 24 hours before the embryos were expected to die from the infection. Moribund infected yolk sacs were harvested from these eggs just before the deaths of the embryos (fig. 1). Uninfected 9-day eggs were also inoculated with P^{32} and reincubated to produce labeled normal yolk sacs. In both cases the yolk sacs were harvested 24 hours after the inoculation of the radioactive phosphate.

17. Moulder, J. W., McCormack, B. R. S. and Itatani, M. 1953, Energy requirements for synthesis of feline pneumonitis virus in isolated chick embryo yolk sac. J. Infect. Dis. **93**: 140–149.

18. Davidson, J. N. 1950, The Biochemistry of the Nucleic Acids, London.

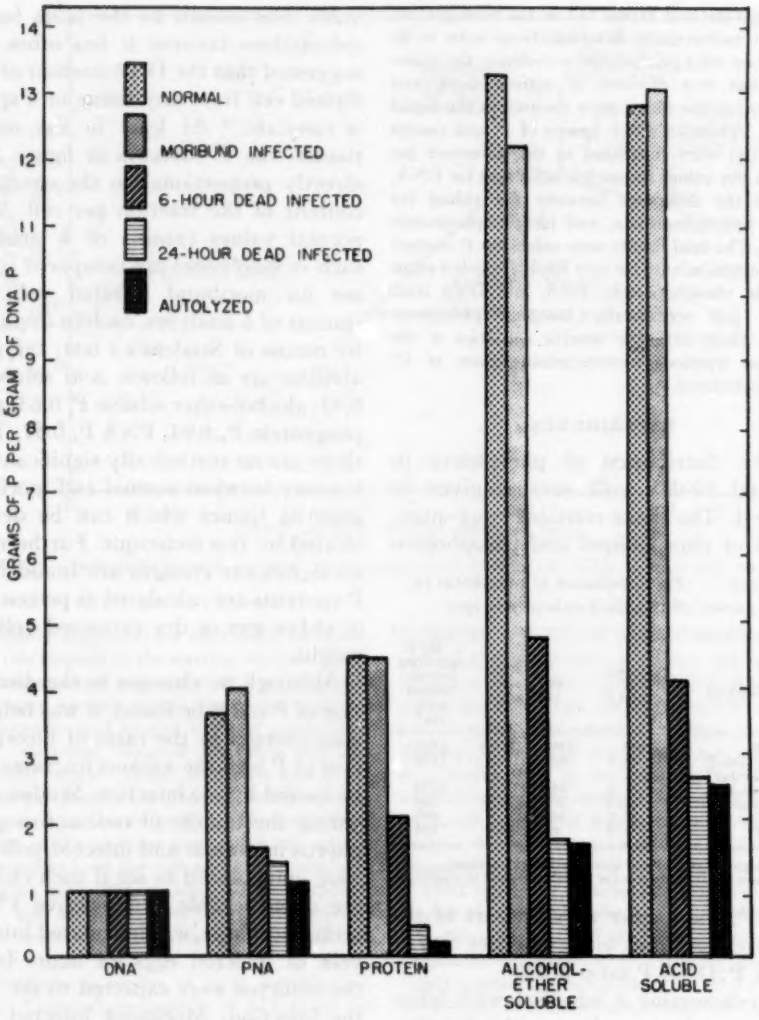

FIG. 2.—The distribution of phosphorus in the various fractions of chick embryo yolk sac.

The course of events following the inoculation of P^{32} into the yolk is complex. We will define *absolute specific activity* (*ASA*) as the counts/minute/gram of P for any chemical fraction or compound. This quantity is directly proportional to the ratio of the grams of P^{32} to the total grams of P in the fraction or compound. It is likely that the sequence of events is as follows. The P^{32} diffuses rapidly through the yolk, becoming evenly distributed throughout it. It enters the cells of the yolk sac more slowly, but the entering P^{32}-labeled phosphate is rapidly brought into equilibrium with the organic acid-soluble P of the cells. Moulder et al[17] have shown that in vitro the yolk sac esterifies 10% of entering

orthophosphate in 30 minutes. At this rate, the organic acid-soluble P would be in equilibrium with entering labeled phosphate in about 10 hours, insofar as any differences in their respective ASA's could be observed. In the living embryo this probably occurs even more rapidly. However, inorganic phosphate is slowly exchanged through the yolk sac with phosphate from other parts of the egg. The ASA's of the yolk P and of the inorganic P of the yolk sac decrease steadily because of this exchange. Data collected by Webster and Ward* support this idea, indicating that there may be a 5-fold reduction in ASA during the 24 hours following the inoculation of P^{32}. The ASA of the inorganic P of the yolk sac thus rises to a maximum soon after the inoculation and steadily decreases thereafter until the P^{32} is distributed through the noncellular portions of the egg. The ASA of the organic acid-soluble P rises until it equals that of the intracellular inorganic P a few hours after the inoculation; thereafter it undergoes the same changes as the ASA of the intracellular inorganic P. Meanwhile, other P-containing compounds in the tissue are being synthesized. This results in the incorporation, direct or indirect, of acid soluble P into them. If the quantities of P in these compounds remain constant for the 24-hour duration of the experiment, as may be assumed for the normal tissue, their ASA's will be approximately proportional to their turnover rate, i.e., to the rates of their synthesis and breakdown.

The ASA's of the fractions varied considerably from experiment to experiment; i.e., the total amount of P^{32} taken up per gram of yolk sac varied. In order to express the specific activities in a form such that the results of one experiment could be compared to those of another, the activity in each fraction in a single experiment was calculated as the percentage of the ASA of the acid-soluble P of that experiment. This value will be called the *relative specific activity* (*RSA*). For any fraction, RSA = (ASA /ASA of the acid-soluble P) $\times$ 100. In all cases the acid-soluble P had a higher ASA than any other fraction. Three different methods[11–13] of isolating inorganic phosphate from the acid-soluble fraction always resulted in an RSA for inorganic P of 100, indicating that the organic acid-soluble P is in equilibrium with the inorganic P at the time of harvesting.

Figure 3 gives the RSA's of the P in the various fractions of normal and moribund infected yolk sacs as the means for three fractionations of each type of tissue. In comparing these values, Student's t test gives the following probabilities: alcohol-ether soluble P, 0.077; phosphoprotein P, 0.75; PNA P, 0.065; DNA P, 0.009. Thus the infection causes a significant increase in the RSA of the DNA P under these conditions; the increases in RSA for the PNA P and the alcohol-ether soluble P are of doubtful significance.

It was considered possible that some of the changes observed in the moribund infected yolk sacs are due to autolysis resulting from the dying condition of the embryo. Investigations were made to determine the effects of permitting the embryos to die from the infection and also to see the effects of autolysis on uninfected yolk sacs. Infected eggs whose yolk sacs were labeled with P^{32} as before were permitted to die from the virus infection and kept at 37 C; their yolk sacs were harvested 6 or 24 hours after the average time of death of their embryos. These were called, respectively, 6-hour dead infected and 24-hour dead infected yolk sacs (fig. 1). In another experiment normal labeled eggs

* Personal communication from R. A. Webster and T. G. Ward.

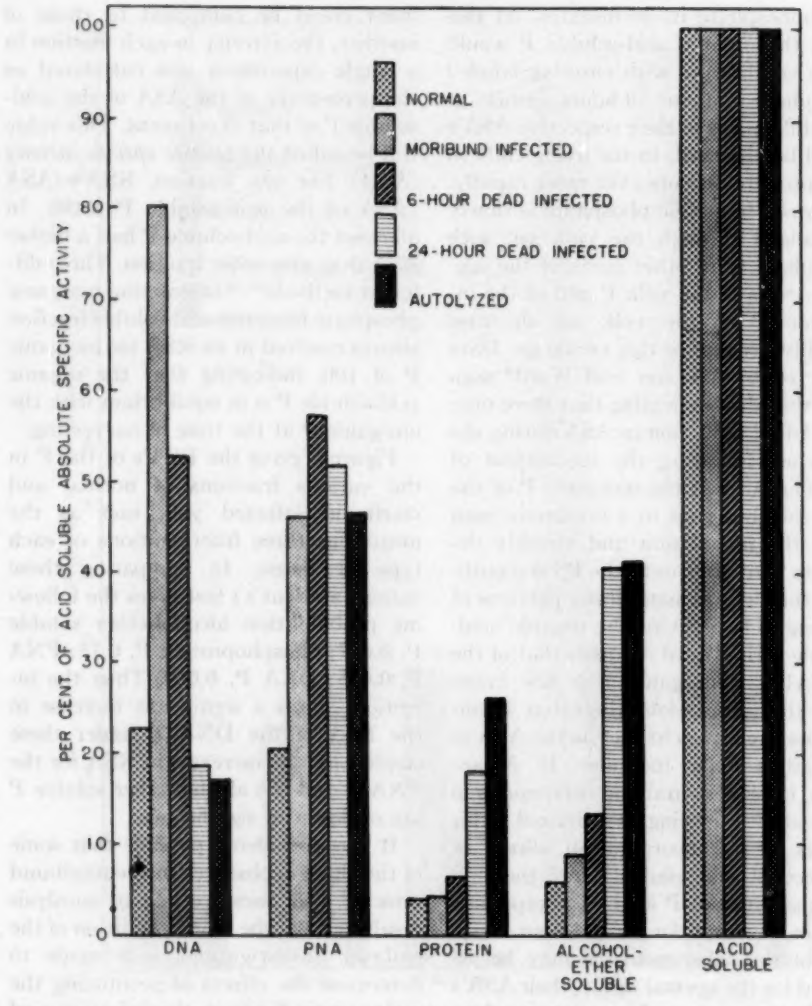

FIG. 3.—The relative specific activities of the phosphorus in the various fractions of chick embryo yolk sac.

were killed by chilling on the tenth day of incubation, 24 hours after P^{32} inoculation. They were then permitted to autolyze for 18 hours at 37 C, at which time their yolk sacs were harvested. The results of the analyses of these yolk sacs are given in figures 2 and 3.

Figure 2 shows that striking changes occur in the amounts of P in the various fractions when infected eggs are permitted to die from the infection, and that these changes parallel closely the autolytic changes occurring in uninfected yolk sacs. Figure 3 shows that incubation after the death of the infected embryos results in changes in the RSA's of the various fractions, which again parallel the changes occurring

during autolysis. Twenty-four hours after the death of the embryos from infection the RSA's of all fractions are very nearly equal to those found after autolysis has progressed for 18 hours in uninfected yolk sacs.

DISCUSSION

The object of these investigations was to throw new light on the mechanisms of animal virus reproduction by studying the changes in the metabolism of chick embryo yolk sac which are caused by infection by feline pneumonitis virus. The virus alone shows no demonstrable metabolic activity.[9] The host tissue's metabolism is, presumably, a well-defined purposeful mechanism whose design enables it to aid in the nourishment of the developing embryo; but the virus-infected tissues demonstrates a new pattern of metabolism, directed toward the production of new virus. The following discussion is concerned with the metabolic pattern of the infected tissue and how it differs from that of the normal tissue. The more basic question of how the virus causes these changes is beyond the scope of this paper.

On the tenth day of incubation the chick embryo yolk sac contains a large amount of phospholipid which may be part of the endogenous energy source.[17] This material has a low turnover rate. It is possible that only a small fraction of the total phospholipid is being synthesized in this stage of the chick's development, and that the rest of this fraction is either metabolically inert or is being used up slowly. The yolk sac also contains a large amount of phosphoprotein whose P moiety is turned over at a very slow rate. Again, this may mean a slow over-all metabolism for the whole phosphoprotein fraction, or it may indicate the presence of a small, highly active protein phosphate, masked by inert phosphoprotein. The latter supposition is in line with the presence in other tissues of a small, very active phosphoprotein fraction.[19] The tissue contains three or four times as much PNA as DNA. The P moieties of both nucleic acids turn over rapidly. The lability of the DNA P is unexpected, since the tissue demonstrates practically no mitotic figures at this time.

If the embryo is killed by chilling and the tissue permitted to autolyze for 18 hours, all of the fractions of the yolk sac lose P, with the possible exception of the DNA fraction. The specific activities of the P in all fractions except the DNA P increase in relation to the specific activity of the acid-soluble P, indicating a continued incorporation of P into these fractions. In the phosphoprotein and alcohol-ether soluble fractions this incorporation seems to occur at a faster rate than in the yolk sacs of living embryos. The DNA appears to remain metabolically inert after embryo death.

If yolk sacs are harvested just before the death of the embryos from feline pneumonitis virus infection, some of these autolytic changes are seen. An increase in the specific activity of the alcohol-ether soluble fraction occurs, which is explicable as a result of autolysis. An increase in the specific activity of the PNA P occurs, which is more difficult to explain. This increase is equal to that occurring during 18 hours of autolysis; it may be due in part to autolytic changes, but evidence which will be presented elsewhere[20] suggests that a specific action of the virus is responsible. The specific activity of the DNA P is

19. Johnson, R. M. and Albert, S. 1952, Evidence of a non-nucleic, protein bound phosphorus with high metabolic activity. Fed. Proc. **11**: 236.

20. Zahler, S. A. and Moulder, J. W. 1953, The incorporation of radioactive phosphate into feline pneumonitis virus in the chick embryo yolk sac. J. Infect. Dis. **93**: 159–165.

increased more than threefold over the normal value. This must be due to the action of the virus, since no parallel increase occurs during autolysis.

If the yolk sacs are harvested at different times following the death of the embryos from the infection, changes in the amounts of P in the various fractions occur which closely parallel those occurring in autolysis. The specific activities of the protein and alcohol-ether soluble P moieties increase to the levels expected after similar periods of autolysis. The specific activity of the PNA P remains at about the level it had attained just before embryo death; there may even be a slight decrease. This may mean that during autolysis the increase in PNA P specific activity occurs very rapidly at about the time of embryo death, after which there is no new synthesis of PNA. An alternative hypothesis, involving the incorporation of P into PNA during autolysis at about the same rate as in normal tissue, and a simultaneous degradation of labile PNA of a high specific activity which is produced in response to the virus, is described in another paper.[20] The specific activity of the DNA P decreases to about the level expected in the uninfected tissue at the time of embryo death or after autolysis. This can be explained by postulating the breakdown of a very active DNA fraction whose production had been induced by the virus; evidence favoring such an interpretation, derived from studies on labeled purified virus, will be presented elsewhere.[20]

SUMMARY

1. The distribution of phosphorus in various chemical fractions of normal chick embryo yolk sac and yolk sac infected with feline pneumonitis virus has been determined. The uptake of orthophosphate labeled with P^{32} into these materials has also been studied. The fractions which were analyzed were acid-soluble, alcohol-ether soluble, phosphoprotein, desoxypentose nucleic acid (DNA), and pentose nucleic acid (PNA).

2. The infection causes no demonstrable change in the distribution of phosphorus in the yolk sac fractions. It does, however, cause the yolk sac to incorporate labeled phosphate into its DNA at a rate far greater than that of normal tissue. The rate of phosphate incorporation into PNA may also increase during infection.

3. Autolysis caused by incubating eggs after they have been killed by freezing or by the virus results in the loss of phosphorus from all fractions of yolk sac, with the possible exception of the DNA. The incorporation of phosphate into the alcohol-ether soluble and phosphoprotein fractions occurs during autolysis at rates at least equal to those occurring in the normal tissue. The incorporation of phosphate into DNA does not occur during autolysis.

THE INCORPORATION OF RADIOACTIVE PHOSPHATE INTO FELINE PNEUMONITIS VIRUS IN THE CHICK EMBRYO YOLK SAC

STANLEY A. ZAHLER* AND JAMES W. MOULDER

From the Departments of Bacteriology and Parasitology and of Biochemistry, The University of Chicago, Chicago 37, Illinois

Previous studies[1,2] have indicated that the energy for the synthesis of feline pneumonitis virus in chick embryo yolk sac is supplied by the aerobic oxidation of endogenous substrates and the concomitant production of organic acid-soluble phosphate esters. Infected yolk sacs, during the 24 hours just preceding the death of the embryos, incorporate phosphorus into their desoxypentose nucleic acid (DNA) fraction much more rapidly than do uninfected yolk sacs. The infection may also cause an increase in the rate of incorporation of phosphorus into pentose nucleic acid (PNA).

This investigation was made to clarify the mode of feline pneumonitis virus reproduction. Virus purified from infected yolk sacs labeled with radioactive phosphorus (P^{32}) was fractionated and compared with the yolk sac pool from which it was prepared. Both DNA and PNA appear to be integral components of the virus, and the nucleic acids of the purified virus have higher specific activities than any chemical fraction of the yolk sac pool from which the virus is isolated.

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* Mr. and Mrs. Frank G. Logan Fellow, 1951. Present address: Department of Bacteriology, University of Illinois, Urbana, Illinois.

1. Moulder, J. W., McCormach, B. R. S. and Itatani, M. 1953, Energy requirements for the synthesis of feline pneumonitis virus in chick embryo yolk sac. J. Infect. Dis. **93**: 140–149.
2. Zahler, S. A. 1953, J. Infect. Dis. **93**: 150–158.

MATERIALS AND METHODS

A description of the virus, eggs, and methods of inoculating and titrating the virus has been given by Moulder and Weiss.[3]

Preparation of purified virus.—A typical protocol for separating feline pneumonitis virus from yolk sac is given below, and any departure from this procedure will be noted in the description of specific experiments. Emulsions of highly infected chick embryo yolk sacs were obtained as described by Moulder and Weiss,[3] except that 0.1 *M* Krebs' phosphate buffer, pH 7.4,[4] was used as a diluent instead of veal infusion broth. About 300 ml of crude infected yolk sac emulsion from 100 eggs were blended in a Waring blendor for 30 seconds and then incubated with 1 mg salt-free crystallized trypsin per ml for 1 hour at 25 C. The digest was cooled to 0 C and centrifuged in an angle centrifuge (Servall SS-1) at 5000×*g* for 1 hour at 0 C. The supernatant, containing large amounts of yolk lipid, was removed as completely as possible, and the virus-containing sediment was suspended in 150 ml of 0.01 *M* Krebs' phosphate buffer, pH 7.4, in 0.154 *M* sodium chloride. To the suspension was then added 1 g of Celite (Johns-Manville Analytical Filter-Aid) per 10 g of yolk sac in the starting material. The mixture was shaken well and after 5 minutes was centrifuged in a horizontal centrifuge (International, size 1) at 300×*g* for 15 minutes at 0 C. The supernatant was removed from the packed Celite sediment and centrifuged at 5000×*g* for 30 minutes to sediment the virus. The virus was next suspended in 150 ml of 0.01 *M* glycyl-glycine, pH 7.4, in 0.154 *M* sodium chloride and centrifuged again at 5000×*g* to reduce the level of inorganic phosphate before fractionation and analysis. The final virus sediment was suspended in 15 ml of the glycyl-glycine-saline, centrifuged in the horizontal

3. Moulder, J. W. and Weiss, E. 1951, Purification and properties of the agent of feline pneumonitis. J. Infect. Dis. **88**: 56–67.
4. Krebs, H. A. and Eggleston, L. V. 1940, The Oxidation of pyruvate in pigeon breast muscle. Biochem. J. **34**: 442–459.

centrifuge for 10 minutes at $300 \times g$, and the final supernatant removed from a small precipitate.

The final virus suspensions contained about 0.1 mg of total nitrogen, exclusive of the glycylglycine, 0.04 mg of total phosphorus, and $10^{7.7}$ chick embryo LD_{50}'s per ml. One LD_{50} contained $10^{-11.5}$ to $10^{-12.0}$ g total nitrogen. Determination of the number of elementary bodies in the suspensions by the counting method of Gogolak[5] indicated that one LD_{50} is equivalent to about 100 elementary bodies. This low value suggests that little virus inactivation occurs during the course of the purification.

The success of the separation procedure depends upon the tryptic digestion. Brown et al[6] have shown that treatment of crude infected yolk sac emulsions with trypsin increases the chick embryo LD_{50} titer two- to fourfold and greatly reduces the amount of sedimentable nitrogen. When the sediment from the trypsin-digested emulsion is treated with Celite in the amounts used here, most of the virus is adsorbed on the Celite and lost, but about 10% remains in the supernatant. When this virus is sedimented and washed it appears to be almost completely free of normal yolk sac components. Examination with light and electron microscopes reveals the presence of many elementary bodies and little cell debris. Rabbit antiserum against normal yolk sac gives no precipitate with the final virus suspensions. Evidence for the presence or absence of yolk sac phosphorus in the final virus suspensions is considered below.

Fractionation, labeled phosphate, and analysis.—Methods for the fractionation and analysis of the virus and for the preparation of the P^{32} are described by Zahler,[3] except that in the fractionation procedure the starting material was two 5.0 ml aliquots of purified virus in glycyl-glycine-saline, instead of yolk sac homogenates in the trichloracetic acid. Single, rather than duplicate, fractionations and analyses of purified virus preparations were made. The procedure results in values for the amounts of P and, where applicable, the specific activity of the P in the following fractions of purified virus: acid-soluble, alcohol-ether soluble, phosphoprotein, PNA, and DNA.

Labeled, purified virus.—In the single experiment in which labeled purified virus was prepared, 7-day embryonated eggs were inoculated into the yolk with $10^{6.7}$ LD_{50}'s of virus, a dose which causes embryo death in 69 to 75 hours. Forty-eight hours after the virus inoculation, 33.5 microcuries of P^{32}, as orthophosphate, were inoculated into the yolk of each living egg. Yolk sacs were harvested 24 hours after the inoculation of P^{32}. These yolk sacs were thus prepared in the same manner as the *moribund infected* yolk sacs of Zahler,[3] except that they were harvested from eggs whose embryos had already died as well as from those whose embryos were moribund. After harvesting, the yolk sacs were treated as in the purification procedure above. This differed from the preparation of the moribund infected yolk sacs in the following respects: the yolk sacs were not washed, they were not chilled immediately, and they were emulsified in 0.1 *M* phosphate buffer instead of being homogenized in trichloracetic acid. A sample of the emulsion was taken for fractionation and analysis. It was frozen at −70 C until the purified virus sample was ready. Then 0.5 ml of it (about 0.167 g of wet yolk sac) was suspended in 9.5 ml of cold 5% trichloracetic acid and fractionated and analyzed at the same time as was the purified virus. Virus was purified from the rest of the yolk sac emulsion in the manner described above.

EXPERIMENTAL

Purification and analysis of feline pneumonitis virus.—Three samples of purified feline pneumonitis virus were analyzed for their phosphorus distribution. Two of these samples were purified from yolk sac pools to which had been added uninfected yolk sacs labeled with radioactive phosphorus. The starting pools were composed of infected unlabeled yolk sacs and uninfected labeled yolk sacs in the ratio of 4:1. The uninfected yolk sacs were prepared by inoculating 7-day eggs into the yolk with 11.2 microcuries of P^{32} as orthophosphate. These yolk sacs were harvested 72 hours after P^{32} inoculation. The presence of radioactivity in any fraction of the purified virus indicated the presence of normal yolk sac P in that fraction. Table 1 shows the losses in infectivity, nitrogen content, and radioactivity during one

5. Gogolak, F. M. 1953, Purification of murine pneumonitis virus from mouse lung. J. Infect. Dis. **92**: 248–253.
6. Brown, A., Itatani, M. K. and Moulder, J. W. 1952, The effect of trypsin and other enzymes on feline pneumonitis virus. J. Infect. Dis. **91**: 184–190.

TABLE 1.—*Separation of feline pneumonitis virus from infected yolk sacs in the presence of normal yolk sacs labeled with P^{32}.*

Fraction	Infectivity		Total nitrogen		P^{32}		Total nitrogen per LD_{50}	P^{32} per LD_{50}
	LD_{50}'s per fraction	Percent of original	Mg per fraction	Percent of original	C.p.m. per fraction	Percent of original	Grams	C.p.m.
Original tryptic digest	$10^{9.88}$	100	405	100	1.01×10^{9}	100	$10^{-9.77}$	$10^{-9.88}$
First high-speed sediment	$10^{9.80}$	83	15.5	3.8	6.30×10^{8}	4.1	$10^{-11.11}$	$10^{-9.40}$
High-speed sediment after Celite adsorption	$10^{9.41}$	11	0.58	0.15	6.90×10^{7}	0.07	$10^{-11.46}$	$10^{-8.97}$

of these purifications. It will be seen that the ratio of counts/minute to LD_{50}'s is decreased by a factor of 100 by the purification process, indicating that about 1% of the purified virus P is of normal yolk sac origin. A 100-fold reduction in the ratio of grams total nitrogen to LD_{50}'s is also obtained. Fractionation of purified virus samples prepared in this manner showed that most of the radioactivity could be isolated in the alcohol-ether soluble fraction of the virus; a small amount was present in the PNA fraction. The other fractions of the purified virus were not detectably contaminated with normal yolk sac P. However, there was insufficient P^{32} in the purified virus to determine quantitatively the extent of contamination in the various fractions.

In the three analyses, the average DNA P content per LD_{50} was 3.3×10^{-14} g. The results of the analyses are given in table 2. The amount of acid-soluble P in the purified virus varied from sample to sample, and there is evidence (see below) that this fraction consists of phosphate absorbed nonspecifically on the virus during the purification procedure. Both DNA P and PNA P were found, although Crocker[7] has reported the absence of PNA in purified meningopneumonitis virus, a closely related organism.

TABLE 2.—*The distribution of phosphorus in purified feline pneumonitis virus.*

Fraction	Grams of P per gram of DNA P			
	Purified virus sample			Mean
	I	II	III	
Acid-soluble P	9.3	13.5	30.6	17.8±6.5*
Alcohol-ether soluble P	9.5	10.6	13.8	11.3±1.2
Phosphoprotein P	2.3	1.5	3.2	2.3±0.5
PNA P	3.1	2.0	2.6	2.5±0.3
DNA P	1.0	1.0	1.0	1.0

* Mean ± standard error.

The distribution and incorporation of phosphorus in labeled purified feline pneumonitis virus.—In one experiment virus was purified from a pool of labeled infected yolk sacs. Both the pool and the virus were analyzed, and the results are given in table 3. The average results for moribund infected yolk sacs, reported in a previous paper,[2] are also given. The method of preparing the pool of yolk sacs differed from the preparation of the moribund infected yolk sacs in several respects. Despite these differences the analytical values were comparable to those obtained with moribund infected yolk sacs, except that some PNA P, and about one-half of the DNA P, remained insoluble in the phosphate buffer and was lost during the emulsification.

The *absolute specific activity* (*ASA*) of the P in any fraction is the counts/minute/gram of P in that fraction. Previous work with this system[2] has shown that

7. Crocker, T. T. 1952, Estimates of particle number, lethal effect, and chemical properties of virus of meningopneumonitis. Fed. Proc. 11: 464–465.

the fraction with the highest ASA in yolk sacs at the time of harvesting is the acid-soluble fraction, and that in this fraction the inorganic phosphate is in equilibrium with the organic phosphate esters. The specific activities of all other fractions have been reported as percentages of the acid-soluble P ASA. This value is the *relative specific activity* (*RSA*), which is equal to (ASA of fractions P/ASA of acid soluble P) $\times$100. In the experiment under discussion, however, the acid-soluble fraction was diluted with inorganic phosphate buffer before the emulsion sample was taken for fractionation. The ASA of this fraction, therefore, could not be used as a basis for calculating the RSA's of the other fractions. Instead, the ASA of the organic acid-soluble P, which had been in equilibrium with the acid-soluble inorganic P before the latter was diluted with buffer, was taken as 100 for calculations of RSA's. The RSA of the PNA P was elevated; this may have been due to autolysis occurring before and after the yolk sacs were harvested.

The RSA's of the P in the various fractions of the purified virus were also calculated as percentages of the ASA of the organic acid-soluble P of the emulsion. Examination of table 3 reveals that the acid-soluble fraction of the purified virus was approximately in equilibrium with the diluted acid-soluble fraction of the emulsion, suggesting that this material was adsorbed by the virus during the purification procedure. The virus alcohol-ether soluble P and phosphoprotein P were approximately in equilibrium with the acid-soluble P of the emulsion before dilution with buffer. The nucleic acid P moieties of the virus had specific activities several times that of the most active fraction of the whole yolk sac at the time of harvesting.

DISCUSSION

If feline pneumonitis virus is purified from labeled yolk sacs which were harvested at about the time of embryo death, not more than 0.3% of the yolk sac P is isolated as virus P. This represents 10 to 15% of the total amount of virus present in the yolk sacs. Virus P, therefore, comprises not more than 3% of the total yolk sac P. Under these conditions the virus DNA P has a specific activity about five times that of the yolk sac acid-soluble P, which is the most active fraction of the yolk sac. Virus PNA P has about four times the specific activity of the yolk sac acid-soluble P, and more than five times the specific activity of the yolk sac PNA P. These data preclude the adsorption of host nucleic acids in any appreciable quantities, and strongly suggest that

TABLE 3.—*Comparison of the distribution and uptake of phosphorus in purified feline pneumonitis virus and the labeled yolk sacs from which the virus was isolated.*

Fraction	Grams of P per gram of DNA P			Relative specific activity		
	Purified virus	Yolk sacs*		Purified virus	Yolk sacs*	
		I	II		I	II
Total acid-soluble P	30.6	127	13.1	17.4†	24.5†	100‡
Inorganic P		115			16.7	100
Organic P		12.0			100	100
Alcohol-ether soluble P	13.8	25.2	12.3	100.3	8.9	8.8
Phosphoprotein P	3.2	9.9	4.5	124	4.2	4.3
PNA P	2.5	3.5	4.1	410	70.5	46.0
DNA P	1.0	1.0	1.0	505	80.4	80.4

* Yolk sacs I represents the yolk sac emulsion from which the labeled virus was isolated. Yolk sacs II represents the mean values obtained for moribund infected yolk sacs by Zahler.[3]

† Relative specific activity = percentage of the absolute specific activity (counts/minute/gram of P) of the organic acid-soluble P of yolk sacs I.

‡ Relative specific activity = percentage of the absolute specific activity (counts/minute/gram of P) of the total acid-soluble P of yolk sacs II.

the virus PNA and DNA are integral parts of the virus particles. Crocker[7] has reported that the closely related meningopneumonitis virus contains no PNA. His purification procedure, which includes suspending the virus in distilled water, may cause the loss of PNA and of virus activity. His elementary body: LD_{50} ratios were 3.6 to 10 times ours, and his suspensions may have contained many inactive (PNA-less?) elementary bodies. The presence of DNA and PNA has been demonstrated by staining techniques in purified feline pneumonitis virus in this laboratory.[3] The specific activities of the virus alcohol-ether soluble and phosphoprotein P moieties are about equal to that of the acid-soluble P of the yolk sacs from which the purification was made. It is interesting to note that when Graham and McClelland[8] purified influenza A virus from the allantoic fluid of eggs which had been inoculated with P^{32}, the virus protein-bound P (including phosphoprotein, PNA, and a small amount of DNA) had a specific activity three times that of the virus phospholipid P. The virus acid-soluble P in our preparation had a specific activity about equal to that of the acid-soluble fraction of the emulsion after dilution with phosphate buffer, suggesting that the virus acid-soluble P was adsorbed from the medium during the purification process.

A satisfactory mechanism for virus growth must explain the following results obtained in this and the previous study:[2] the great increase during infection of the yolk sac DNA P specific activity, which is more than five times that which can be explained by the presence of highly labeled virus DNA P; the decrease in the specific activity of the DNA P of infected yolk sacs when eggs are incubated after the embryos have died from the infection; and the fact that the specific activities of the virus nucleic acid P moieties are several times that of the most active fraction of the yolk sacs from which the virus is isolated.

A working hypothesis which is consistent with the data presented, can be offered to explain these results but is subject to modification in the light of future work. In the following discussion the specific activity of the acid-soluble P of the yolk sac 24 hours after P^{32} inoculation will be taken as 100.

In the uninfected egg the specific activity of the inorganic P of the yolk sac reaches a maximum a few hours after the P^{32} inoculation; then, as the P^{32} is diluted with unlabeled phosphate from the rest of the egg, the specific activity of the intracellular inorganic P decreases. The maximum value for the specific activity of this fraction is several hundred; it is reached 3 to 12 hours after the P^{32} inoculation. The specific activity then decreases until the radioactive P is evenly distributed throughout the noncellular portions of the egg, at which time it is 100. During the 24 hours of the experiment, acid-soluble P is being incorporated into other fractions of the yolk sac at various rates, rapidly in the cases of the nucleic acids, and more slowly in the cases of the alcohol-ether soluble and phosphoprotein fractions. Death of the embryo, either by chilling or from virus infection, when followed by incubation at 37 C, results in the continued incorporation of acid-soluble P into the alcohol-ether soluble, phosphoprotein, and PNA fractions; the tissue DNA remains inert after embryo death. During this period of autolysis P is lost from all fractions, with the possible exception of the DNA.

When P^{32} is inoculated into an egg

8. Graham, A. F. and McClelland, L. 1950, The uptake of radioactive phosphorus by influenza virus A (PR8 strain). Canadian J. Res. **28E**: 121–134.

which is infected with feline pneumonitis virus 24 hours before the embryo is expected to die from the infection, the specific activity of the inorganic acid-soluble P undergoes the same changes as in the normal yolk sac. At the time of the inoculation most of the yolk sac cells are infected with the virus. The infection causes a great increase in the rate of production of DNA by the tissue. The newly formed virus-induced DNA is labile. Only a small fraction of it becomes incorporated into newly synthesized virus, and this process causes it to become relatively inert metabolically. The rapid synthesis of new DNA ceases perhaps 12 hours before the death of the embryo. All of the stable virus DNA has been formed at this time, although the number of infectious virus particles continues to increase until the time of embryo death. The P moiety of the virus DNA has the same specific activity as the acid-soluble P from which it was formed—about 500. The labile DNA whose synthesis was induced by the virus breaks down gradually; at the time of embryo death some of it is still present in the cells. Since the specific activity of the virus DNA P is about 500, if we assume that all of the virus-induced DNA P has the same specific activity, it follows that about 10% of the total DNA of moribund infected yolk sacs is virus-induced; the other 90% is the normal tissue DNA. If the egg is incubated after the death of the embryo the labile virus-induced DNA breaks down rapidly. Furthermore, about 80% of the virus DNA breaks down, accompanied by a 5-fold reduction in virus titer during the 24 hours following embryo death. At the end of this time, then, a very small amount of the total DNA of the tissue is virus DNA; most of it is normal tissue DNA, which is metabolically inert after the death of the embryo. The specific activity of the total DNA P in the yolk sac is hardly affected by the small amount of virus DNA P present, and is about the same as that of the normal tissue DNA after autolysis.

The high specific activity of the PNA P in the moribund infected yolk sacs can be explained in a similar manner, except that the incorporation of acid-soluble P into normal tissue PNA continues after embryo death just as in the autolysis of uninfected yolk sacs. If this is correct, 6 to 12% of the total PNA P in moribund infected yolk sacs is virus-induced.

The hypothesis outlined above parallels the current ideas on bacteriophage multiplication[9,10] in that it postulates that the nucleic acids of the virus are laid down early in the infection, before the appearance of the infectious virus. Feline pneumonitis virus, however, is not as efficient as the phages in that it induces the production of far more nucleic acid than is required for the newly synthesized virus. Again drawing from the phage work, it is possible that the non-nucleic acid P moieties, in this case, phospholipids and phosphoproteins, combine with the nucleic acids during the last few hours of the growth cycle to form infectious elementary bodies.

SUMMARY

1. Feline pneumonitis virus has been purified from infected chick embryo yolk sacs by a simple, rapid technique involving emulsification, tryptic digestion, the adsorption of normal yolk sac components on Celite, and several cycles of differential centrifugation.

9. Cohen, S. S. 1951, The synthesis of nucleic acid by virus-infected bacteria. Bact. Rev. 15: 131–146.

10. Evans, E. A., Jr. 1952, Biochemical Studies of Bacterial Viruses, Chicago.

2. Purified virus contains desoxypentose nucleic acid, pentose nucleic acid, phosphoprotein, and alcohol-ether soluble phosphorus compounds. The preparations also contained acid-soluble phosphorus which was probably adsorbed by the virus during the purification procedure.

3. Virus was purified from yolk sacs which had been labeled with radioactive phosphorus. The specific activities of the alcohol-ether soluble and phosphoprotein P moieties were about equal to that of the acid-soluble P of the yolk sacs from which the virus was isolated, while the virus nucleic acid P moieties had specific activities several times as high.

4. A hypothesis is offered to explain the high specific activities of the P moieties of the nucleic acids of infected yolk sacs and the purified virus. It postulates that the virus induces an increase in the rates of nucleic acid formation by the tissue during the period ending several hours before the death of the chick embryo from the infection, while the acid-soluble P of the yolk sac has a very high specific activity. Not all of the virus-induced nucleic acid is incorporated into the newly synthesized virus, and the unincorporated nucleic acid is catabolized rapidly.

TRANSMISSION EXPERIMENTS WITH MAEDI

BJÖRN SIGURDSSON, PÁLL A. PÁLSSON, AND ANNA TRYGGVADÓTTIR
From the Institute for Experimental Pathology, The University of Iceland, Keldur, Reykjavík, Iceland

A chronic pneumonia of sheep in Iceland was described recently.[1] This disease, which is called maedi, is undoubtedly infectious, although the epidemiological pattern as observed here does not give any decisive indication as to the mode of spread. The disease is believed to be spread by contact, presumably through droplet infection, but the possibility of other modes of spread and even of the presence of an intermediate host or vector should certainly be kept in mind.

It was pointed out in the earlier publication[1] that microscopically visible organisms which could be incriminated as the cause of the disease have not been found in infected tissue. Such organisms have been searched for in young lesions, before the disease has progressed very far, and also in more advanced cases, when the size of the lungs has increased greatly or up to two to five times their normal weight. It is suspected that the disease is caused by a virus.

From experience in the field it seems that the disease has a very long incubation period, extending to approximately two to three years. The onset is insidious, and the course of the clinical phase of the disease is very protracted. Mortality is very high and it is doubtful whether recoveries ever occur.

This paper describes some experiments designed to elucidate the mode of infection in maedi. Although the evidence obtained is by no means complete in any respect, it is considered worthwhile to publish the results, as transmission work with this disease can no longer be continued here. The reason for this is that the sheep stock in all maedi-infected areas in Iceland has now been destroyed in an attempt to eradicate the infection, and it is not considered safe to continue transmission experiments with maedi under these conditions.

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1. Sigurdsson, B., Grímsson, H. and Pálsson, P. A. 1952, J. Infect. Dis. **90**: 233–241.

RESULTS

The 1st experiment to be described was begun in October 1945. Fifteen lambs, divided in 3 groups of 5 each, were used. The 1st group received a 5% suspension of a mixture of infected lungs and mediastinal lymph nodes. Five ml of the suspension were injected directly into the lung and 2 ml into each nostril of each sheep. The animals were kept in a light chloroform anesthesia during the inoculation.

The 2nd group received an 0.1% suspension of another typical maedi lung and lymph node in exactly the same way as the 1st group. The maedi lungs used for inoculating these sheep both showed typical maedi lesions and produced no growth on the ordinary bacteriological mediums.

When the lambs were inoculated they were about 5 months old and had been purchased shortly before from an area where maedi had never been known to occur.

The 3rd group of 5 lambs was kept as controls.

All the animals were kept in separate pens, but in the same shed, where airborne infection could have passed from

one group to another. They were kept in their pens continuously while the experiment lasted, and they were all killed in August 1947, 22 months after the inoculation. At the time when the experiment was terminated all the animals were in an excellent condition and showed no signs of disease.

On autopsy all the lungs were found to be macroscopically quite normal, and they were not subjected to further scrutiny, although this might have been desirable in view of later findings.

The 2nd experiment was begun in January 1948. This was designed to test whether healthy lambs could be infected by contact with active cases of maedi. Two groups (A and B) of 5 lambs each were kept in contact with a total of 6 cases of maedi which were rotated between the 2 groups for a period of 3 months. One of the experimental groups, A, was fed a supplement of 7.5 ctg of copper sulfate and 1.5 ctg of cobaltous chloride twice a week, while no such supplement was given to group B. Besides the 2 groups A and B, which we attempted to infect with maedi, a 3rd group, C, was kept as controls. The 3 groups were kept in separate pens in the same shed. As in the 1st experiment, isolation between the groups was inadequate for preventing the spread of air-borne infection between the animals. The sheep were bled monthly and white and red blood cells were counted in the usual way. Hemoglobin was determined in a photoelectric colorimeter. The animals were weighed once a month.

Two sheep died from infections other than maedi during the experimental period, both of them from group B. They died after 17 and 21 months, respectively.

Three sheep were killed with symptoms of advanced maedi after 27, 28 and 30 months, respectively. They had all been ill for several months and they all belonged to group A, which received the supplement of microelements. The remaining animals were slaughtered in August 1950, 32 months after the experiment was begun.

The results of the experiment as far as maedi morbidity is concerned may be seen from table 1.

It will be seen that 4/5 in group A, 2/3 in group B and 3/5 in the control group contracted the disease. It is felt that the disease was definitely most severe in group A, which also received copper and cobalt.

The figures for the variation of the number of blood cells and the concentration of hemoglobin as observed are difficult to interpret. There were rather striking variations in the numberof red cells and the concentration of hemoglobin during the experimental period. These variations occurred rather synchronously in the 3 groups. Since the intended controls also contracted the disease there were no actual control animals in the experiment, and the variations are therefore very difficult to interpret. The extensive data will therefore not be published here.

The figures for the leucocyte counts are also somewhat difficult to interpret. The results for the 3 groups are shown in figure 1. It will be seen that one

TABLE 1.—*Results of experiment 2.*

	Sheep no.	Weight of lungs in grams	Infected with maedi	Specific deaths; months after exposure
Group A—maedi and microelements	51	956	+	
	62	965	+	30
	63	905	+	27
	76	930	+	28
	67	325	–	
Group B—maedi	52	600	+	
	61	390	–	
	64	Died from an intercurrent infection		
	66			
	68	530	+	
Group C—controls	69	1275	+	
	70	330	–	
	71	500	+	
	72	310	–	
	77	860	+	

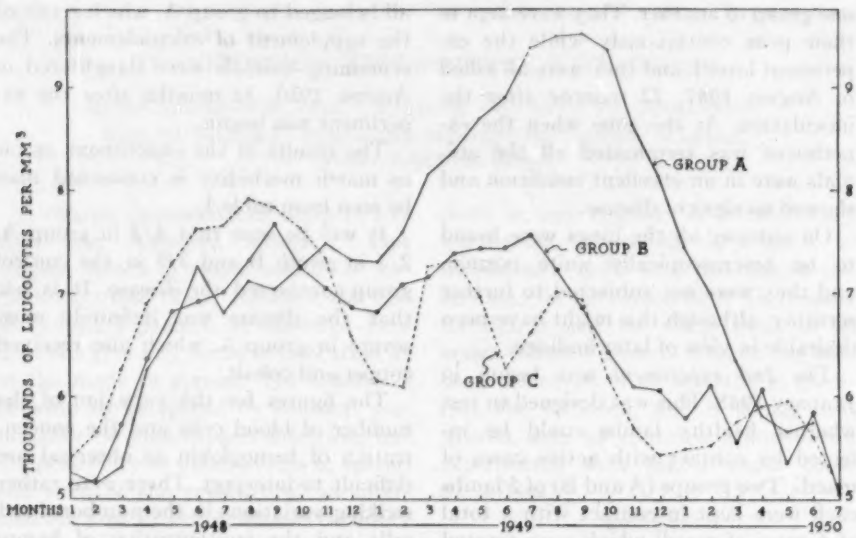

FIG. 1.—The number of white blood cells found in the 3 groups of sheep in the 2nd experiment. The figures plotted are the average numbers for each of the 3 groups.

of the groups developed a high level of leucocytes about 1 year before the onset of clinical disease. This group (A) received a copper and cobalt supplement, and 3 of the animals belonging to it were the only ones which died from maedi during the experimental period.

Figure 2 shows the variations in the white cell counts in the individual animals in group A. It will be seen that the 3 animals which became severely ill and died (62, 63, and 76) were the ones that exhibited a leucocytosis for at least a year before the disease had become clinically manifest. It should also be pointed out that in all 3 cases did the leucocyte count begin to fall again about the time when signs of lung involvement became clinically noticeable. By the time the process in the lungs had begun to threaten the life of the animals the leucocyte count was down to normal.

The 2 remaining animals in this group (51 and 67) did not show a comparable rise in their leucocyte counts. One of these sheep (51) was at autopsy found to have maedi, but the other (67) was considered normal. The leucocyte curve for no. 51, which contracted maedi, rose slightly above its previous level about a year before the animal was killed, but it is by no means certain that the curves for the 2 last-named sheep differ at all in this respect.

The 5 animals from groups B and C which at autopsy showed maedi changes in the lungs (see table 1) did not show any clear evidence of previous leucocytosis. These animals had much more slowly progressing lesions in the lungs.

The present experiment does not in itself prove that the disease was transmitted, as the controls also came down. As already mentioned, however, it was realized from the beginning that isolation facilities were not adequate for preventing the spread of air-borne infection between the groups. There is, however, ample evidence from other sources that the disease is transmitted from one sheep to another, and we believe that such has also been the case here, and

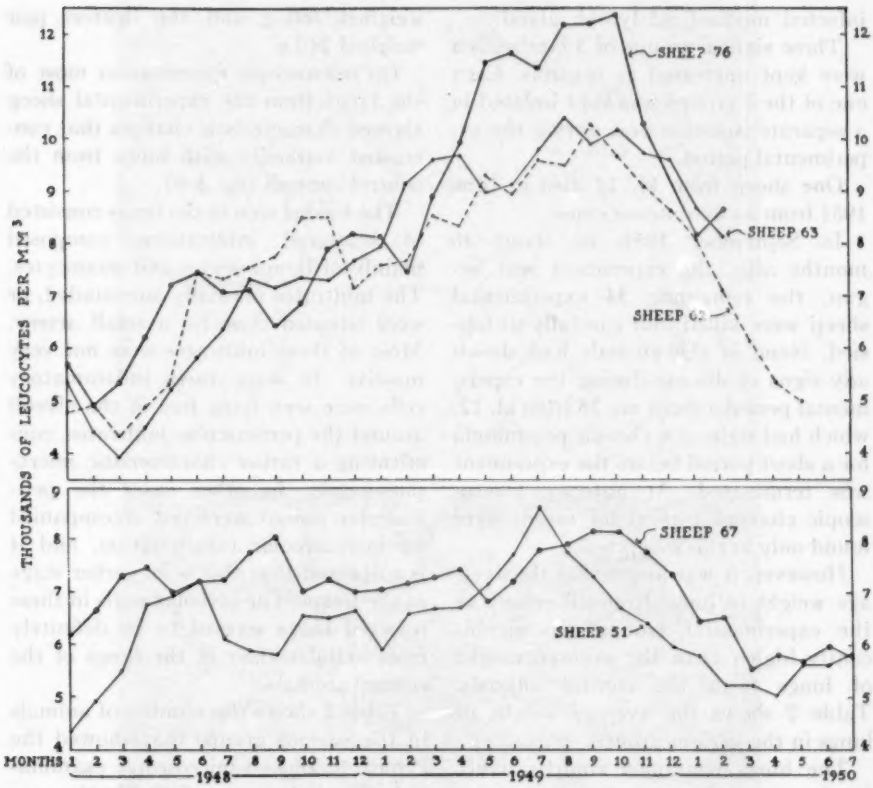

FIG. 2.—The number of white blood cells found in each of the 5 sheep in group A. For clarity of presentation the 3 animals which showed a definite leucocytosis are placed separately. Those were the same animals which contracted severe maedi infection.

that the clinical incubation period varied from approximately 25 to 35 months.

The 3rd experiment was begun in March 1949 and comprised 7 groups of 5 lambs each. This time facilities were available for keeping the different groups in adequate isolation. The lambs were about 9 months old when the experiment was begun. Transmission of maedi to the experimental animals was attempted by the following methods:

A. Contact with cases of maedi.—Two of the groups, called kl. 2, and kl. 10, were kept in contact with active cases of maedi for a period of 3 months. Four sick animals were sequentially placed in each isolation unit one at a time.

B. Contamination of drinking water with "infected" faeces.—One group (kl. 6) was on 6 occasions during the first 3 months of the experiment given drinking water contaminated with faeces from 4 cases of active maedi. Twenty g of ground faeces per 8 liters of water was given each time.

C. Injection of maedi tissue.—One group (kl. 12) was inoculated intravenously (5 ml), intrapulmonally (2 ml) and intranasally (1 ml) with a 5% suspension of a mixture of maedi lung and

infected mediastinal lymph gland.

Three similar groups of 5 lambs each were kept untreated as controls. Each one of the 7 groups was kept isolated in a separate isolation unit during the experimental period.

One sheep from kl. 12 died in June 1951 from an extraneous cause.

In September 1951, or about 30 months after the experiment was begun, the remaining 34 experimental sheep were killed and carefully autopsied. None of the animals had shown any signs of disease during the experimental period, except no. 78 from kl. 12, which had signs of a chronic pneumonia for a short period before the experiment was terminated. At autopsy macroscopic changes typical for maedi were found only in this sheep.

However, it was found that the average weight of lungs from all or any of the experimental groups was significantly higher than the average weight of lungs from the control animals. Table 2 shows the average weight of lungs in the various groups.

The lungs contained comparatively few lung worms, and, in our experience, lungs of healthy, young sheep that have been kept for long periods of time in strict isolation are remarkably light and more uniform in weight than lungs from sheep living under natural conditions. This is illustrated in the 15 control sheep in the present experiment which showed a lung weight of 302 g ± 11. The heaviest lungs in these sheep weighed 380 g and the lightest pair weighed 240 g.

On microscopic examination most of the lungs from the experimental sheep showed characteristic changes that contrasted markedly with lungs from the control animals (fig. 3–6).

The lesions seen in the lungs consisted of scattered infiltrations composed mainly of lymphocytes and monocytes. The infiltrates normally surrounded, or were situated close to, a small artery. Most of these infiltrates were not very massive. In some cases inflammatory cells were seen lying free in the alveoli around the perivascular infiltrates, constituting a rather characteristic micropneumonia. In other cases the perivascular lesions were not accompanied by intraalveolar inflammation, and it is suspected that this is an earlier stage of the lesion. The alveolar walls in these infected lungs seemed to be definitely more cellular than in the lungs of the control animals.

Table 2 shows the number of animals in the various groups that showed the typical lesions on microscopic examination. The lesions were found in the contact groups, in the injected animals, and also in the group that was fed faeces from infected sheep.

The conclusion seems inescapable, that the lesions found in the lungs, combined with the increase in weight of the infected lungs, compared with the controls, is due to some agent transmitted in our experiments. It seems most likely

TABLE 2.—*Findings in the lungs of the sheep in experiment 3.*

Groups	Kind of treatment	Number of animals	Average weight of lungs in grams	Probability that difference is by chance*	Typical microscopic lesions in:†
kl. 2, kl. 10	Contact infection	10	352.5 ± 16	<0.02	8/9
kl. 6	Faeces per os	5	394 ± 20	$\ll 0.01$	4/5
kl. 12	Injected with maedi tissue	4	502.5 ± 75	$\ll 0.01$	3/4
kl. 5, kl. 9, kl. 11	Controls	15	302 ± 11		0/15

* Probability that the weight in each group is not significantly different from the weight of lungs in the control groups.
† The denominator indicates how many pairs of lungs were examined from each group. The numerator shows how many pairs of lungs were found showing the changes described in the text.

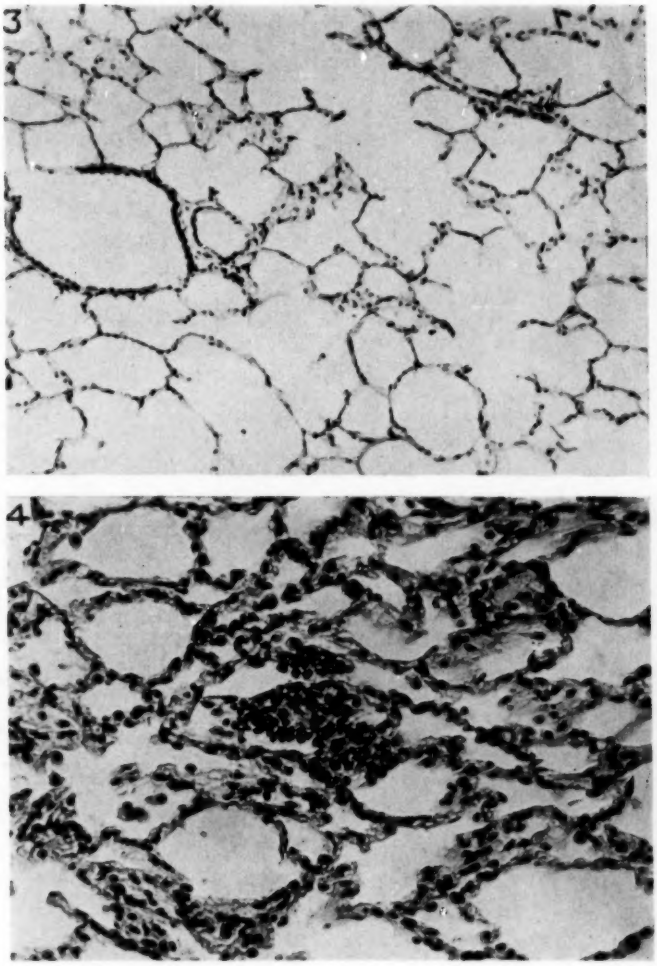

FIG. 3.—Section of normal lung from a healthy control animal. ×125.
FIG. 4.—Small periarteriolar infiltrations with few intraalveolar cells. ×255.

that the changes in the lungs are early maedi lesions, as they are all of the same kind and qualitatively the changes are distinctly of the maedi type.

The 4th experiment was begun in November 1951. The purpose of this was to confirm, if possible, some of the findings from the 3rd experiment, and also to learn how soon microscopic lesions would develop in the lungs after the injection of infected material.

Ten lambs were injected with a suspension of typical maedi lungs at the age of 6 months. Under a light chloroform anesthesia each lamb received 2 ml into the lung, 2 ml into the nose and 5 ml intravenously. Another similar group of 10 lambs received an exactly

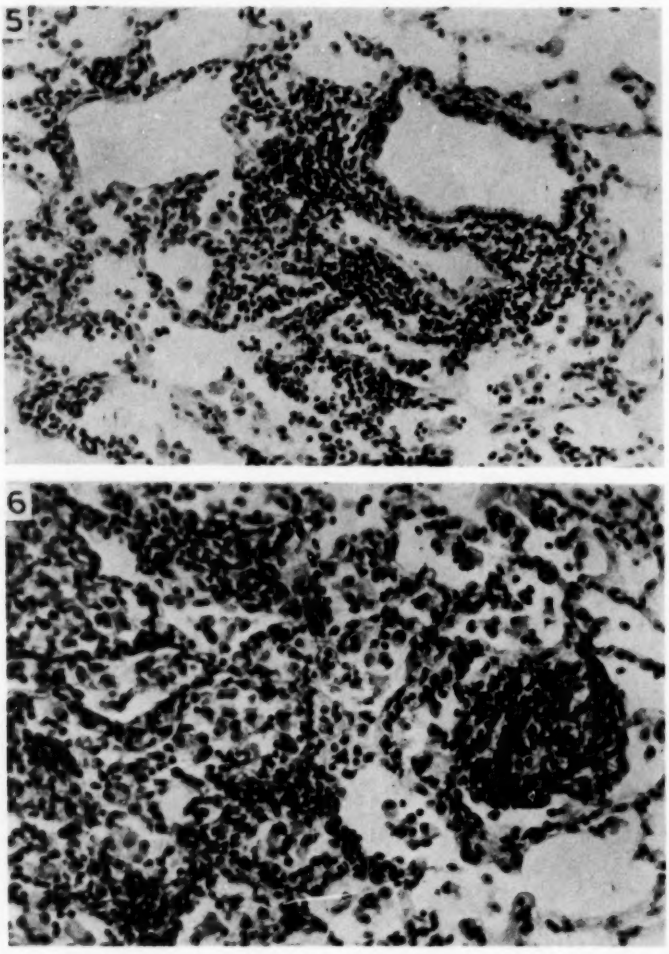

FIG. 5.—A periarteriolar infiltration with a considerable number of cells in the surrounding alveoli. ×200.

FIG. 6.—The micropneumonia has developed further than in the preceding pictures. The perivascular infiltrations are more massive and the alveoli are crowded with mononuclear cells. ×250.

corresponding treatment with a suspension from a normal lung. The animals were kept in 4 separate isolation units, 5 in each.

By the time these experiments were begun it was known that all transmission work with maedi would have to end not later than October 1952. The experiment was therefore planned so that the last animals should be autopsied before that time.

At varying intervals after the inoculation, groups of 4 lambs—2 inoculated with maedi and 2 inoculated with normal material—were slaughtered and carefully autopsied. The lungs were weighed, and the left lung fixed by distending it with 10% formalin infused

TABLE 3.—*The number of typical lesions found in lungs of sheep from experiment 4.*

	Number of microscopic lesions found in lung after:				
	10 days	30 days	90 days	210 days	285 days
Maedi					
A	0	12	80	2	0
B	19	3	4	18	0
Controls					
A	2	2	0	0	0
B	0	0	0	0	0

through the main bronchus. After at least 7 days of fixation 6 pieces were cut from each lung at predetermined sites in the lung. From these pieces tissue sections were examined systematically under the microscope and typical lesions were counted. The total area of these sections from each lung examined under the microscope was approximately 10 to 12 cm.^{2}

The lesions counted were distinct perivascular or parabronchiolar infiltrations and/or light infiltrations with similar cells in the alveoli. Table 3 shows the outcome of these counts, A and B representing the 2 sheep in each group.

It seems likely that the findings 10 days after the inoculation should be considered an unspecific result of the inoculation which was both intravenous and intrapulmonal. The individual lesion, a perivascular infiltration and a micropneumonia, is not pathognomonic for maedi. However, the apparent accumulation of such lesion in the lungs of the maedi-inoculated animals, which were slaughtered 30 or more days after the inoculation, does suggest that they are due to the inoculation, although the number of experimental animals is not large enough to prove this.

The 5th experiment was designed to test whether the sheep ked (*Melophagus ovinus*) plays a role in the transmission of maedi. The ked is the only ectoparasite of sheep in Iceland which is so widespread that it could be suspected of transmitting maedi in all the areas where the disease has been prevalent.

Four groups of 6 lambs each were used for this experiment. Each group was kept in adequate isolation while the experiment lasted. All the groups were deloused with dips containing benzenehexachloride before the experiment was begun. The lambs were then exposed as indicated in table 4.

The experiment was begun in May 1951 and was terminated in September 1952, 15 months later, at which time all the sheep were killed and autopsied. All the lungs were macroscopically normal and no significant variation in weight of lungs was found between the various groups. The left lung was fixed by intrabronchial infusion with 10% formalin and examined as described under the 4th experiment.

As may be seen from table 4 the results were almost completely negative, although a few lesions were found in 3 sheep.

DISCUSSION

The experimental results reported

TABLE 4.—*Number of typical lesions found in lungs of sheep from experiment 5.*

The groups were exposed as follows:	Number of lesions found in lungs from sheep					
	Sheep A	Sheep B	Sheep C	Sheep D	Sheep E	Sheep F
In contact with ked-free cases of maedi 5/25/50 to 6/30/51	0	0	0	0	0	0
In contact with ked-infested cases of maedi 5/11/51 to 6/18/51	0	4	0	0	0	0
Infested with keds from cases of maedi 5/23/51 to 6/18/51	0	0	2	0	3	0
Controls	0	0	0	0	0	0

here suggest subjects for future study, but scarcely allow definite conclusions as to the mode of transmission and the pathogenesis of maedi.

It is concluded, however, that maedi was definitely transmitted by contact in the 2nd experiment reported here. The incubation period was about 2 years. Three animals died prostrate with the disease 27, 28 and 30 months, respectively, after the first contact with maedi, and when the remaining animals were autopsied 32 months after the first contact, actively progressing lesions were found in 6 more sheep. The group of sheep which had been intended as controls also contracted the disease. At that time facilities for keeping the animals in strict isolation, which would preclude the transmission of air-borne disease between the groups, were not available, and it is also possible that the person who took care of the experimental flock might have carried, for instance, sheep keds between them, although this seems unlikely.

In this experiment one of the groups was fed a supplement of copper and cobalt in order to explore the possibility that a deficiency of these elements might be a factor aggravating the symptoms of maedi. The converse seemed rather to be the case, as the symptoms of maedi proved to be most severe in the group that received the supplement.

Those animals which received a supplement of copper and cobalt developed a marked leucocytosis that lasted for almost a whole year in three out of four cases. The leucocytosis was most marked in the three animals which developed the most severe disease, and which died before the experiment was terminated. No explanation is offered at the present time for the fact that leucocytosis developed only in the copper-cobalt group but not in the animals in the other groups that contracted maedi.

There is no evidence that a nutritional deficiency of copper or cobalt is present in sheep fed the ration in use at this institute, but no microelement analyses were done on the sheep in this experiment. The concentration of hemoglobin was approximately equal in the group receiving the microelements and in the other groups.

Analyses on blood from other sheep kept at this institute after the 2nd experiment was terminated have always shown a normal value for copper.

The results obtained in the 3rd and 4th experiments suggest that maedi may develop in the lungs extremely gradually in the form of scattered, minute infiltrations. Whether these lesions progress continuously until the well recognized gross lung lesions have developed, or whether the disease progresses in discontinuous steps, cannot be decided at the present time.

The results from the 3rd experiment seem to prove that maedi may be transmitted by injecting infected tissue intranasally, intrapulmonally and intravenously. One of the sheep so injected developed definite maedi lesions and two others developed lesions that are believed to be the first recognized stage of maedi.

The negative results of the first experiment where transmission by inoculation was also attempted may be explained by the circumstance that the experimental animals were slaughtered only 22 months after the experiment was begun, and then only the macroscopic appearance of the lungs was examined. The negative results could also be explained by assuming that the particular lungs used in these experiments did not contain the active agent in a sufficient quantity or in an active form.

The experiments (exp. 5) designed to test the role of the sheep ked (*Melophagus ovinus*) in the transmission of

maedi barely suggest that this possibility should be explored further if opportunity arises.

Transmission work with maedi will not be continued now because of the danger of spreading the infection from the laboratory after the surrounding districts have been cleared of the disease.

Experimental work with this disease necessarily progresses rather slowly because of the long incubation period, and because of the fact that, as far as is known, only sheep are susceptible, which has precluded the use of other animals.

Repeated attempts to transmit maedi to the small laboratory animals have constantly met with failure.

SUMMARY

Maedi is a chronic progressive pneumonia of sheep in Iceland, and is suspected to be of virus origin. This paper reports on the transmission of the disease to healthy sheep in the laboratory.

The incubation period until macroscopically distinct lesions develop and clinical symptoms are manifest is about two years.

It is believed that an early microscopically visible stage, in the form of scattered minute infiltrations in the lungs, mainly perivascular in site, was recognized in this work.

A prolonged leucocytosis beginning about a year before signs of maedi were noticeable was observed in sheep to which maedi was successfully transmitted, and which received a feeding supplement of copper and cobalt, but not in other sheep which also contracted maedi but which did not receive such a supplement.

THE INFLUENCE OF ADJUVANTS ON THE ELIMINATION OF SOLUBLE PROTEIN ANTIGENS AND THE ASSOCIATED ANTIBODY RESPONSES

DAVID W. TALMAGE† AND FRANK J. DIXON

From the Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

One of the modes of action of adjuvants used to enhance the immune response has been thought to be the slowing of the absorption, destruction and elimination of the antigen. In the case of the Freund adjuvant used with bacterial antigens, this retention of antigen at the injection site has been demonstrated.[1,2] The present report deals with the quantitative determination of the ability of water-in-oil emulsions (modified Freund adjuvant) and alum to hold soluble protein antigens at subcutaneous injection sites. I^{131}-labelled protein antigens prepared as previously described[3] have been used with these adjuvants, and the amount of antigen remaining at the injection site has been followed by external measurement of the γ radiation. The associated antibody responses have also been followed by appropriate serum antibody determinations.

EXPERIMENTAL PROCEDURE

Antigen preparations were injected into the subcutaneous tissue of the dorsum of the left hind feet of 6 groups of albino male rabbits weighing 2 to 2.3 kg as follows:

1. Six received 1 cc of a 5% solution of I^{131}-labelled bovine γ globulin, Armour and Company lot no. C 904, (I*BGG) in 0.85% saline.
2. Six received 1 cc of a 5% water-in-oil emulsion of I*BGG prepared by mixing in a cold micro Waring blender for 5 minutes 10 parts of a 10% I*BGG solution in saline, 9 parts Bayol F and 1 part Arlacel A, according to Freund and coworkers.[4]
3. Four received 1 cc of an alum precipitate preparation of I*BGG made by adding 10 ml of 1% alum solution to 80 ml of a 0.15% solution of I*BGG and then adding $N/10$ sodium hydroxide to maximum turbidity, a modification of the technique described by Heidelberger and Kendall.[5]
4. One received 1 cc of a 5% solution of I^{131}-labelled crystalline egg albumin (I*Ea) in 0.85% saline.
5. Five received 1 cc of a 5% water-in-oil emulsion of I*Ea prepared as for group 2.
6. One received 1 cc of an alum precipitate preparation of I*Ea made by adding 5 ml of 1% alum solution to 90 ml of 0.15% solution of I*Ea and then adding $N/10$ sodium hydroxide to maximum turbidity.

Each antigen preparation contained approximately 100,000 counts per minute per injection as determined by a well-type γ counter‡ just after injection.

The I* antigen at the injection site was measured immediately after injection and at 1- to 2-day intervals thereafter by placing the entire injected foot and lower leg into the well-type counter. The noninjected right hind leg was also counted at each determination to give a control value, which included both background counts and any radioactivity attributable to antigen circulating in the blood. The count in the noninjected leg was subtracted from the count in the

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† Present address: Dept. of Medicine, University of Chicago, Chicago 37, Illinois.

1. Freund, J. 1947, Ann. Rev. Microbiol. **1**: 291–308.
2. Herdegen, M., Halbert, S. P. and Mudd, S. 1947, J. Immunol. **56**: 357–364.
3. Talmage, D. W., Dixon, F. J., Bukantz, S. C. and Dammin, G. J. 1951, J. Immunol. **67**: 243–255.
4. Freund, J., Thomson, K. J., Hough, H. B., Sommer, H. E. and Pisani, T. M. 1948, J. Immunol. **60**: 383–398.
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‡ Kindly loaned by The Texas Company, Belaire, Tex.

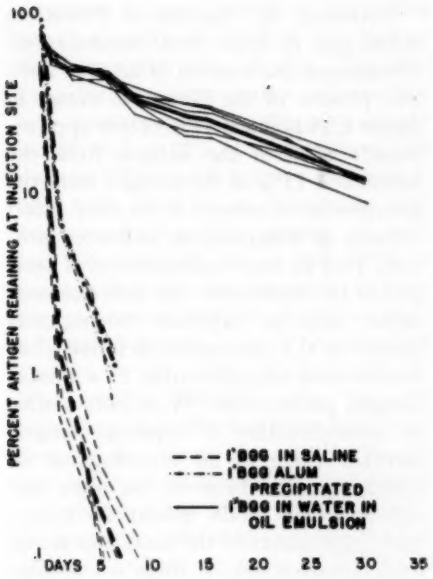

FIG. 1. I*BGG remaining at subcutaneous injection site in rabbits. Fine lines in each group represent values for individuals and heavy lines the group averages.

injected leg to give a final value for the antigen remaining at the injection site.

In groups 1 and 2 receiving I*BGG in either saline or water-in-oil emulsion the amount of antigen in the blood was traced by determining the amount of protein bound I^{131} in the blood, according to the method previously described.[5] The antibody responses of animals injected with antigen in saline and in emulsion were followed by measuring the antigen precipitating capacity of the serum at several intervals after injection as previously described.[6]

RESULTS AND DISCUSSION

From figures 1 and 2 it is apparent that the water-in-oil emulsion held relatively large amounts of both I*BGG and I*Ea at the injection site for long periods of time. There was an average loss of 30% of the I*BGG and 20% of the I*Ea from the injection sites within the first 24 hours. Following this relatively rapid loss, the disappearance approached an exponential rate with an average half-life of approximately 14 days for both antigens. Since most of the initial, rapid loss of I*BGG from the injection site can be accounted for by the appearance of protein bound radioactivity in the serum (see discussion of figure 3 below), it seems likely that this early loss of radioactivity from the injection site represents actual release of antigen from the emulsion and not simply a dissemination of the emulsion. The initial relatively rapid release of antigen from the emulsion indicates either that first, a portion of the antigen is not firmly bound in the emulsion, or that second, the conditions in the tissues are most favorable for breaking down the emulsion and releasing antigen immediately following injection. The subsequent relatively slow, exponential rate of antigen release does not

FIG. 2.—I*Ea remaining at subcutaneous injection site in rabbits. Fine lines represent values for individuals and the heavy line the average for the emulsion group.

6. Talmage, D. W. and Maurer, P. H. 1953, J. Infect. Dis. **92**: 288–300.

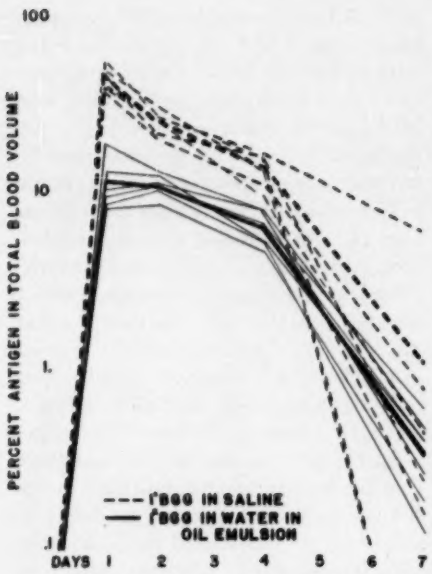

FIG. 3.—Blood levels of I*BGG after subcutaneous injection in rabbits. Fine lines represent values for individuals and heavy lines the group averages.

appear to be influenced by time, the fibrous tissue reaction which develops about the emulsion depot, or by the appearance of circulating antibody.

Both antigens in saline solution rapidly diffused from the injection site. Only 1% of the I*BGG remained after 2 days and less than 1% of the I*Ea remained after one day (fig. 1 and 2). As will be discussed in the case of I*BGG, the antigen in saline gained rapid access to the blood and equilibrated with the body fluids much as if it had been given intravenously. The slightly more rapid dissemination of I*Ea can probably be attributed to its smaller molecular size.

Alum precipitation had a slight binding effect on I*BGG, holding 7% at the injection site after 2 days and 1% after 7 days (fig. 1). There was, however, no appreciable retention of the alum precipitated I*Ea at the injection site in the single animal studied (fig. 2).

Following the injection of I*BGG in saline and in water-in-oil emulsion, we determined the amount of labelled antigen present in the blood. As shown in figure 3, 24 hours after injection approximately 50% of the antigen from the saline and 12% of the antigen from the emulsion were present in the total blood volume as water-soluble iodinated protein. This 24-hour concentration of antigen in the blood after the subcutaneous saline antigen injection corresponds closely to the concentration found after intravenous administration of a similar antigen preparation.[3] With both routes of administration of aqueous antigen solution there is an equilibration of labelled protein between the intra and extravascular fluids within 24 hours, with approximately the same amount in each compartment. If there is a similar equilibration of the antigen which escapes from the emulsion depot, one would expect 12% of the emulsified antigen to be in the extravascular fluid and 12% in the intravascular fluid 24 hours after injection. This total of 24% of the antigen in the intra and extravascular fluids is approximately the amount seen to disappear from the emulsion injection site within the first day. A relatively slow loss of antigen from the blood is seen during the next 3 days for both groups of animals. This loss has been described as the nonimmune elimination of antigen.[3] However, 4 days after injection the antigen disappears more rapidly from the blood, presumably as a result of antibody production, and this has been called the immune elimination or catabolism of antigen.[3,7,8] With the

7. Dixon, F. J., Bukantz, S. C., Dammin, G. J., and Talmage, D. W. 1953, The Nature and Significance of the Antibody Response, edited by A. M. Pappenheimer, Jr., New York, Columbia University Press, p. 170.
8. Dixon, F. J. and Talmage, D. W. 1951, Proc. Soc. Exper. Biol. & Med. **78**: 123–125.

exception of one animal in the saline group, no antigen could be detected in the blood after the 7th day. The prolonged nonimmune antigen elimination rate demonstrated by this one animal is seen in 5 to 10% of rabbits given this dose of I*BGG and is invariably associated with little or no detectable antibody production. The absence of antigen after the 7th day in the blood of animals injected with emulsion, in spite of a constant liberation of antigen from the injection site, is to be expected in view of the circulating antibody (fig. 4). The antigen liberated after the 7th day most probably combines with antibody in the tissues surrounding the injection site and is rapidly catabolized.

In figure 4 and table 1 the serum antibody concentrations of 4 rabbits making a good antibody response to I*BGG in saline are compared with 4 rabbits from the group receiving I*BGG in water-in-oil emulsion which survived more than 100 days. The μg I*BGG nitrogen precipitated by 1 cc of antiserum at the 80% precipitation point, as previously described,[6] are plotted on a logarithmic scale against time from the injection of antigen on an arithmetic scale. In response to the saline injection, antibody was detectable first following the elimination of all measurable antigen 7 days after injection. Maximum antibody concentrations are found about 3 days later, the first points plotted on this graph, and are followed by a rapid drop in serum antibody during the next 16 days (table 1). This rate of antibody decline approaches the half-life of passively administered antibody, indicating little production of antibody following the peak concentrations.[7,9,10]

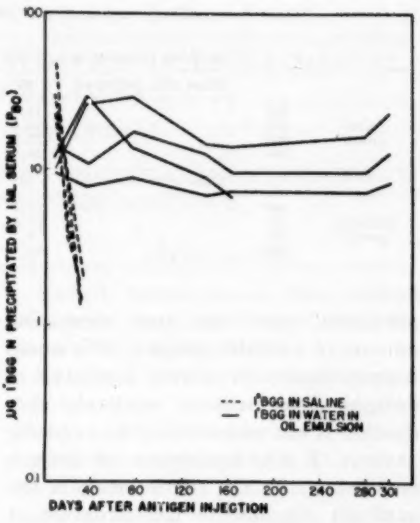

FIG. 4.—Serum anti-BGG concentrations following subcutaneous injection of I*BGG in saline and in water-in-oil emulsion. All lines represent values for individual rabbits.

As might be expected from earlier observations,[4] the antibody response to the I*BGG in emulsion was maintained at a relatively high level for a long period. Although the initial antibody concentrations determined 10 days after injection of emulsified antigen were somewhat lower than those of the saline injected animals, these concentrations were maintained with moderate variations for 301 days after injection (fig. 4, table 1), at which time the determinations were discontinued.

If the observed rate of loss of I*BGG from the emulsion depot during the first 30 days after injection (fig. 1) can be assumed to continue for the next 271 days it is apparent that an extremely small amount of antigen, less than 0.025 μg I*BGG, would remain at the injection site at the time of the last antibody determination 301 days after injection. Thus, there appears to be a relatively constant output of antibody

9. Dixon, F. J., Talmage, D. W., Maurer, P. H. and Deichmiller, M. P. 1952, J. Exper. Med. **96**: 313–318.
10. Dixon, F. J., Talmage, D. W. and Maurer, P. H. 1952, J. Immunol. **68**: 693–700.

TABLE 1.—*Antibody responses to I*BGG in saline and in water-in-oil emulsion.*

		Antibody expressed as μg I*BGG N precipitated by 1 ml serum P_{+6}[a]						
		Days after injection	10	16	20	26		
Saline group	892		15.6	7.4	3.7	1.8		
	895		30.8	15.2	6.4	3.1		
	896		22.6	10.2	4.5	1.5		
	909		26.8	11.2	7.0	3.0		
		Days after injection	10	38	77	139	164	280	301
Emulsion group	340		13.7	10.8	17.2	12.6	9.3	9.3	13.0
	341		11.6	28.8	13.3	8.7	6.7	Died	
	342		9.2	7.4	8.8	6.9	7.2	7.0	8.2
	343		10.2	26.1	28.8	14.4	13.8	16.6	23.2

associated with an ever decreasing amount of available antigen. This would suggest that even at very low levels of antigen concentration, antibody production is not proportional to available antigen. If the persistence of antigen is necessary for the continuation of the antibody response in this situation, it would appear that the cells involved in antibody synthesis either utilize the antigen more efficiently as time goes on or that the concentration of antigen is not a limiting factor in antibody production under these conditions. On the other hand, it may be that the constant level of antibody production maintained over 301 days represents the output of γ globulin by cells which have become enzymatically adapted to the antigen and are able to synthesize antibody independently of antigen.[10,11]

SUMMARY

Administration of soluble protein antigens in water-in-oil emulsion results in a prolonged retention of antigen at the injection site. Rate of antigen elimination from the injection site has a half-life of approximately 14 days. Alum precipitation of protein antigens causes only slight retention of antigen.

The antibody response to emulsified protein antigens is relatively constant for at least 301 days, while the response to these antigens in aqueous solution diminishes rapidly after 10 days.

11. Burnet, F. M. and Fenner, F. 1949, The Production of Antibodies, London, MacMillan.

FURTHER STUDIES ON SUPERINFECTION IN EXPERIMENTAL CAVINE BRUCELLOSIS

I. EARLY SUPERINFECTION

E. H. KELLY, ARTHUR N. GORELICK, SIDNEY J. SILVERMAN, AND WERNER BRAUN
From Chemical Corps, U. S. Army, Washington 25, D. C.

In a previous study (Pollack et al, 1952) we have reported the results of attempts to superimpose infection with *Brucella suis* upon established infection caused by *Brucella abortus*. Guinea pigs infected with *Br. abortus* for 4 to 26 weeks were, with rare exceptions, refractory to infection with *Br. suis*, but resistance to superinfection was much less marked in animals which had been infected for only 2 weeks. Of these guinea pigs, one-half yielded *Br. suis* when sacrificed 5 weeks after superinfection; the remaining animals showed lesions typical of *Br. suis* infection but the organism was not recovered. This suggested that a modified infection might have occurred in which *Br. suis* organisms, after initial dissemination, were rapidly eliminated from the tissues.

It appeared, therefore, that a more detailed study of superinfection during the early stages of *Br. abortus* infection might yield information on the factors determining whether the second infection will become established. The present report describes such a study, in which animals were infected with *Br. suis* at short intervals after infection with *Br. abortus*. The possible occurrence of a transitory infection with *Br. suis* was investigated by sacrificing groups of animals at varying times after superinfection. No attempt was made to follow the pathologic changes in detail, the criterion of infection being in all cases recovery of the organism.

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EXPERIMENTAL

Except where noted, the methods used were those described in the earlier paper (Pollack et al, 1952).

The plan of the experiment was as follows. Two hundred and fifty-five guinea pigs of the Hartley strain (of both sexes, weighing 350 to 450 g) were infected with *Br. abortus* subcutaneously in the right groin. On the same day 40 of these and 20 additional uninfected animals received *Br. suis* subcutaneously in the left axilla. Similar groups of *Br. abortus*-infected and control animals were reinfected with *Br. suis* at 5, 10, 15, and 20 days after infection with *Br. abortus*. Of each group, 10 superinfected animals and 5 *Br. suis* control guinea pigs were sacrificed at 7, 14, 21, and 28 days after superinfection. The remaining 55 *Br. abortus*-infected animals served as controls for the primary infection and were sacrificed at intervals described below.

Because of the magnitude of the study, it was necessary to perform it in two parts; first the 0-, 5-, and 10-day infection intervals were tested, and then the 15- and 20-day intervals. This arrangement necessitated the use of 2 separate *Br. abortus* inoculums and 5 separate *Br. suis* inoculums, as well as 2 separate batches of animals. An attempt was made, by turbidimetric standardization of the suspensions of infective organisms, to use the same numbers of organisms for each group but this was not always successful. The doses chosen for infection were the

same as had been used in the earlier experiment: 1600 organisms of the carbon dioxide-requiring *Br. abortus* strain 6232 and 30 organisms of the streptomycin-resistant PSIIIksr strain of *Br. suis*. The actual doses used are indicated in figure 1.

The schedule of the experiment was so arranged that autopsies were done thrice weekly in the first part and twice weekly in the second part of the experiment. Only one group of 5 *Br. abortus* controls was sacrificed each week; they served as controls for the animals with which they were sacrificed and for those examined 2 days earlier or later.

A rather wide range of tissues was cultured in order to obtain data on the distribution of the respective organisms in the host. The animals to be sacrificed were bled from the heart and electrocuted. Approximately 0.5 ml of blood was cultured on an agar plate and the rest used for agglutination studies.

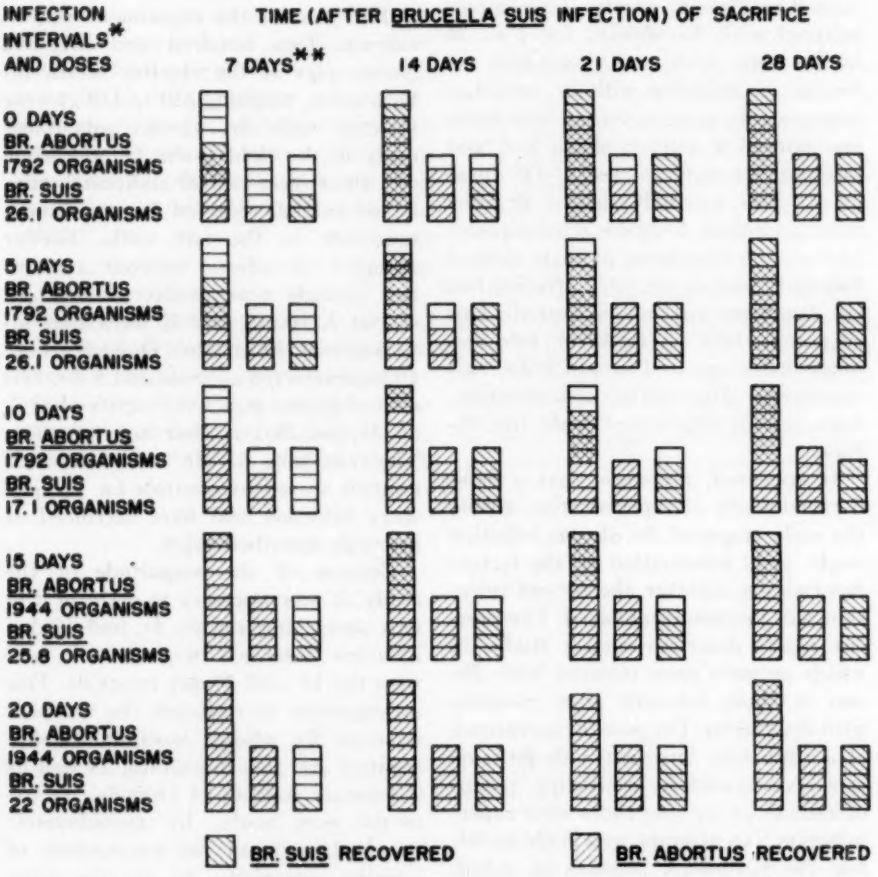

FIG. 1.—Isolation of brucellae from guinea pigs infected with *Br. abortus* and superinfected with *Br. suis*.

* Interval between primary infection with *Br. abortus* and superinfection with *Br. suis*.

** From left to right the bars represent the superinfected group, the *Br. abortus* controls, and the *Br. suis* controls.

Cultures were made of triturated spleen, liver, epididymis or uterus, lung, and the following lymph nodes: right inguinal, left axillary, mesenteric, and bronchial. In addition, a small amount of bone marrow was spread on an agar plate. All cultures from doubly infected animals were made in duplicate, on plain 2-1 agar (Braun, 1950), and on 2-1 agar containing basic fuchsin (1/100,000) to inhibit the growth of *Br. suis*. Cultures from control animals were plated on the appropriate agar with or without dye. All cultures were incubated for 4 days at 37 C, the plain plates in air, the dye plates in candle jars to permit growth of *Br. abortus*. The use of a *Br. suis*-inhibiting dye permitted direct detection of the presence of *Br. abortus* without the tedious and uncertain task of differentiation between the 2 species by inspection of colonial morphology; the latter procedure is, however, applicable to certain strains. In preliminary tests and on check plates made from control animals, *Br. suis* was never found to grow on the dye medium, while *Br. abortus* grew very well. The streptomycin resistance of *Br. suis* was not used routinely for differentiation since the ability of *Br. suis* to grow without added carbon dioxide sufficed to distinguish it from *Br. abortus* 6232 c. The marked strain was used only as an additional precaution in case there might be evidence of transformation from one species to another. A prior titration of the streptomycin-resistant strain of *Br. suis* had shown its virulence to be approximately the same as that of its parent strain PSIIIk.

RESULTS AND DISCUSSION

The experiment yielded information both on the degree to which establishment of *Br. suis* infection is influenced by a prior *Br. abortus* infection and on the course of early cavine brucellosis as determined by cultural methods. Figure 1 contains a general summary of results, showing the recovery of *Br. suis* and *Br. abortus* from all animals. In the figure each block represents a guinea pig, and each bar a group of 9 or 10 which were infected and sacrificed together. The cross hatches indicate whether the animal yielded *Br. abortus*, *Br. suis*, both, or neither. By adding the number of blocks showing both organisms to the number showing only one, the total number of animals yielding either organism can be obtained. It is apparent from the figure that infection with *Br. abortus* greatly reduced the susceptibility of the animal to infection with *Br. suis* and that this effect became evident within 5 to 10 days after the original infection. It is probable that the marked difference in recoveries of *Br. suis* between the 5- and 10-day reinfection groups may be attributed in part to the lower infecting dose which the latter received.

The resistance to *Br. suis* engendered by *Br. abortus* becomes more apparent if one compares the number of controls from which *Br. suis* could be recovered with the total number of animals yielding this organism (alone or with *Br. abortus*) in the reinfected groups, regardless of time of sacrifice. This tabulation appears in table 1, from which it may be seen that the recovery of *Br. suis* from animals given both organisms simultaneously did not differ from that

TABLE 1.—*Effect of duration of* Br. abortus *infection on the isolation of* Br. suis *from guinea pigs infected simultaneously or consecutively with both species.*

Interval between infections (days)	Superinfected animals		*B. suis* controls	
	Ratio*	Percent	Ratio*	Percent
0	30/40	75	15/20	75
5	27/40	68	17/20	85
10	12/40	30	13/19	68
15	14/40	35	16/20	80
20	2/39	5	15/20	75

* Number of animals yielding *Br. suis* (alone or with *Br. abortus*) over total animals cultured. One quarter of the animals in each group were sacrificed at 7, 14, 21, and 28 days after infection with *Br. suis*.

observed in the controls. When administration of *Br. suis* was delayed for as little as 5 days, the percentage of *Br. suis*-infected animals in the control groups noticeably exceeded that in the reinfected groups and further delay increased the difference between the two groups.

Another factor of interest was the distribution of the respective organisms in the tissues of the animals. In the group receiving both organisms simultaneously, the distribution of *Br. suis* within the animals that became infected with this organism did not differ from that in the controls. Beginning with the 5-day infection interval, infection with *Br. abortus* appeared definitely to retard the spread of *Br. suis* within the animals which became superinfected. By 4 weeks after superinfection, however, differences between the *Br. suis* isolations from most of the reinfected animals and from the controls were inapparent; at this time any animal showing *Br. suis* was likely to yield it from most of the tissues cultured. The 20-day infection interval group was the only one in which the spread of the second organism appeared to be suppressed for 4 weeks. The *Br. suis* infection appeared to facilitate the spread of *Br. abortus* during the first weeks after simultaneous infection with both organisms, although by 4 weeks there was no difference between the *Br. abortus* isolations from the doubly infected animals and from the controls. Apart from this group, infection with *Br. suis* did not affect the development of *Br. abortus* infection.

The effect of the infection interval on the distribution of *Br. suis* is shown graphically in figure 2, which presents a comparison of the percent of tissues yielding *Br. suis* in the reinfected and control animals. In the figure the different times of superinfection are represented by separate lines, and the intervals between reinfection and sacrifice by points on the lines. All controls are added together and presented on a single line, on which each point represents 25 guinea pigs. At each point all tissues cultured from every animal in the group are added together. Although as noted above in most groups *Br. suis* eventually spread in individual animals which became superinfected to equal the distribution in the controls, the smaller number of animals yielding *Br. suis* results in quite marked differences between control and reinfected groups.

Serologic studies on these animals included titrations of agglutinins and of complement. The latter titrations revealed no significant differences between controls and superinfected animals. The agglutination studies are summarized in table 2, in which all animals of each group are represented, whether or not they developed the second infection. The results are of interest principally in confirming that low agglutinin titers may be associated with active infection in guinea pigs. The addition of a second infecting organism

TABLE 2.—*Average agglutinin titers of guinea pigs infected with* Br. abortus *followed, after varying intervals, by* Br. suis.

Superinfected animals			*Br. suis* controls		*Br. abortus* controls	
Interval between infection	Days infected*	Average agglutinin titers†	Days infected	Average agglutinin titers†	Days infected	Average agglutinin titers*
0	28	38	28	70	26	5
5	33	115	28	70	33	19
10	38	140	28	69	38	50
15	43	165	28	55	43	130
20	48	258	28	230	48	572

* Days after *Br. abortus* infection; animals had been infected with *Br. suis* 28 days before sacrifice.
† Reciprocal of the average of the titers of all animals.

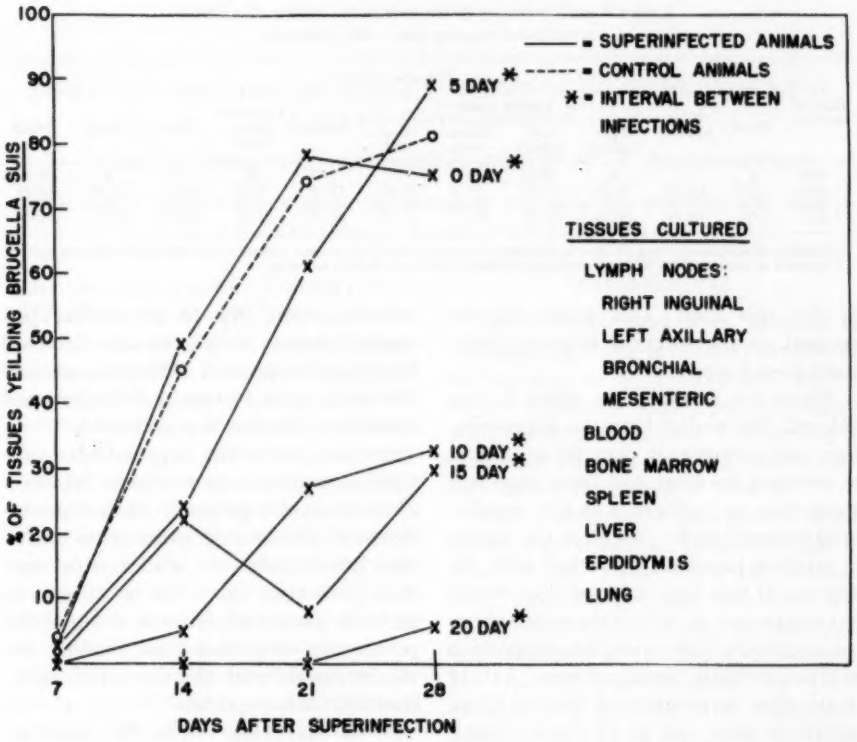

FIG. 2.—Percent of tissues yielding *Br. suis* in guinea pigs infected with *Br. abortus* and *Br. suis*.

appears to have a slight stimulatory effect on the average agglutinin titer in the early stages of infection with *Br. abortus* but not in the later groups, in which fewer animals developed the second infection. No correlation between agglutinin titer at time of sacrifice and occurrence of superinfection could be detected in individual animals.

In addition to the study of superinfection, the experiment yielded information on the progress of *Br. suis* infection in guinea pigs. In table 3, all *Br. suis* control animals sacrificed at each interval (5 groups of 4 or 5, each group having been infected at a different time) are added together and the distribution of *Br. suis* in their tissues is presented. From the data it is apparent that, using an inoculum of this size, it is necessary to wait 4 weeks before sacrifice in order to detect infection in all animals.

In considering the results of this experiment it is important to realize that two effects of time were tested: first, the interval elapsing between successive infections, and second, the time between superinfection and sacrifice. Each markedly affected the outcome.

It is apparent from the results that the first time effect tested, i.e., the interval between infections, is a critical factor in superimposing *Br. suis* upon *Br. abortus* infection. Infection of only 5 days' duration did not prevent superinfection with *Br. suis*, but by 10 days after infection with *Br. abortus* a considerable proportion of the guinea pigs was refractory to infection with the dose

TABLE 3.—*Distribution of* Br. suis *in the tissues of animals sacrificed at varying times after infection.*

Time of sacrifice	Tissues cultured										
	Blood	Bone marrow	Lymph nodes				Spleen	Uterus or Epididymis	Liver	Lung	Total†
			Right inguinal	Left axillary	Mesenteric	Bronchial					
7 days	0	0	0	8/25	0	0	1/25	0	0	0	8/25
12 days	2/21*	13/24	10/24	22/24	6/22	10/24	18/24	4/22	13/23	11/19	22/24
21 days	7/21	21/25	21/25	21/25	18/24	22/24	22/25	11/24	21/25	22/25	22/25
28 days	7/21	19/23	24/24	22/23	23/24	24/24	24/24	12/23	20/23	20/22	24/24

* Number of animals showing *Br. suis* in the respective tissue over total animals cultured; contaminated cultures omitted
† Number of animals from which *Br. suis* was isolated over total animals cultured.

of *Br. suis* used. This proportion increased as the interval between infections grew longer.

When the second time effect is considered, the period between superinfection and sacrifice, it may be seen that, in contrast to what had been expected, there was no indication of any accelerated removal of *Br. suis* from the tissues of animals previously infected with *Br. abortus*. If this had occurred, one would have expected to isolate the superinfecting organism from a higher proportion of those animals sacrificed soon (7 to 14 days) after superinfection than of those sacrificed later (21 to 28 days). Space does not permit detailed presentation of the results, but, in general, the opposite was true; the number of animals in any one group yielding *Br. suis* increased with the elapsed time following superinfection.

Possible mechanisms which might account for the resistance to superinfection engendered by infection with brucellae are discussed in the next paper of this series (Kelly et al, 1953, accompanying paper).

SUMMARY AND CONCLUSIONS

1. Bacteriologic studies of guinea pigs superinfected with *Brucella suis* after primary exposure to *Brucella abortus* revealed little resistance to the second organism in animals which had been infected for 5 days at the time of superinfection. By 10 days after primary infection resistance was detected in a small proportion of the animals and this proportion increased as the interval between infections was extended.

2. Sacrifice of the superinfected animals at weekly intervals after injection of the second organism revealed no evidence of accelerated elimination of *Br. suis* when compared with control animals receiving only this organism. In general, when sacrifice was delayed the percentage of guinea pigs yielding *Br. suis* increased with the time after infection with this organism.

3. In this experiment the level of serum agglutinins in animals superinfected soon after primary infection was higher than in the control animals receiving either organism, but when superinfection was delayed, the administration of the second organism did not appear to affect the average agglutinin titer.

4. A study is presented of the dissemination of *Br. suis* cells in animals receiving only this organism.

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FURTHER STUDIES ON SUPERINFECTION IN EXPERIMENTAL CAVINE BRUCELLOSIS

II. THE EFFECTS OF DIFFERENT COMBINATIONS OF *Brucella* SPECIES

E. H. KELLY, ARTHUR N. GORELICK, SIDNEY J. SILVERMAN, AND WERNER BRAUN
From Chemical Corps, U. S. Army, Washington 25, D. C.

In previous papers (Pollack et al, 1952; Kelly et al, 1953) we have reported experiments in which guinea pigs infected with *Brucella abortus* were superinfected after varying intervals with *Brucella suis*. These studies showed that the second infecting organism could be isolated with ease from animals infected with *Br. suis* simultaneously or at short intervals after the original infection. When the interval between infections ("infection interval") was increased to 2 weeks, however, only half the animals yielded *Br. suis*.

In the experiment to be described the effects of different combinations of species of *Brucella* were tested. The infection intervals and time between the second infection and sacrifice were selected on the basis of the earlier work.

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EXPERIMENTAL

The methods used were, in general, the same as those previously employed except that, as described below, the selective mediums varied in accordance with the organisms used. The strains of organism injected and the time intervals tested are shown in table 1.

Each combination and time interval shown was tested in 10 guinea pigs of the Hartley strain. Control animals were included for each infecting dose and were sacrificed at appropriate intervals in groups of 10, except the controls for the PSIIIksr infection, for which groups of 5 animals were considered sufficient in view of the amount of information on this strain available from the previous experiment.

The animals were bled and sacrificed 4 weeks after superinfection. The following tissues were cultured: right inguinal, left axillary, and bronchial lymph nodes, spleen, liver, and bone

TABLE 1.—*Strains of organisms injected and time intervals tested.*

1st infection (right groin)	Dose organisms/animal	Duration of first infection (days)	2nd infection (left axilla)	Dose organisms/animal
Br. melitensis 4247w	40	0		24
	40	5	*Br. suis* PSIIIksr	27
	40	15		27
	40	5	*Br. melitensis* 4247wsr	32
	40	15		32
Br. suis PSIIIk	34	5	*Br. suis* PSIIIksr	33
	31	15		33
	34	4	*Br. abortus* 6232csr	4800
	31	15		4800
Br. suis PSIIIksr	33	5	*Br. melitensis* 4247w	40
	26	15		40

"sr" designates streptomycin-resistant strains.

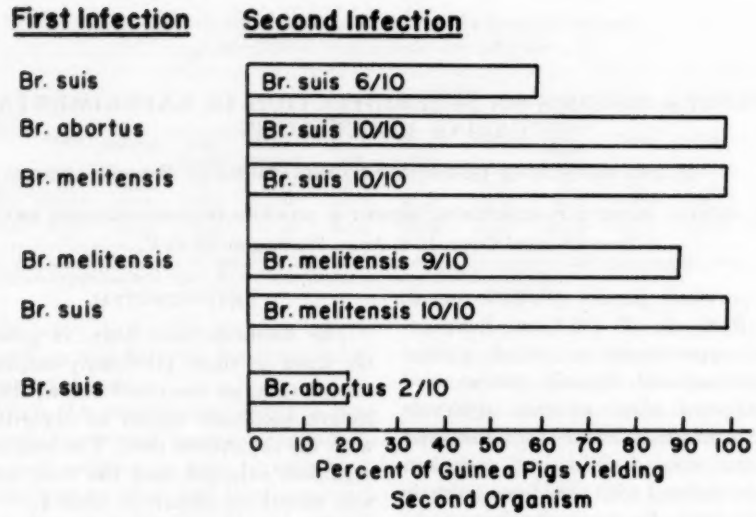

FIG. 1.—Recovery of *Brucella* species superimposed on brucellosis of 5 days duration.
The length of the bar indicates the percent of guinea pigs yielding the second organism; the numbers following the name of the second organism represent the number yielding the superinfecting organism over total superinfected.

marrow. The mediums and conditions of incubation used for cultures were varied to suit the particular combination of organisms under test. Agar containing streptomycin was used to detect the streptomycin-resistant strain in each case. *Br. suis* and *Br. abortus* were distinguished by the streptomycin resistance of the latter and its inability to grow without added carbon dioxide. For detecting the presence of *Brucella melitensis* it had been expected that its ability to grow on agar containing basic fuchsin, which inhibits *Br. suis*, would suffice. Unfortunately, although stock cultures of *Br. melitensis* grew almost quantitatively on the dye agar, the organism as recovered directly from the animal did not, and the results of melitensis-suis combinations were therefore not entirely satisfactory. In the "homologous" superinfection groups (suis-suis and melitensis-melitensis) only the streptomycin resistance of the second infecting organism was employed for identification; in animals yielding the second organism, the presence of the original infection could only have been detected by testing a large number of colonies for streptomycin sensitivity. Since the object of the experiment was to determine whether superinfection would occur, such efforts were not deemed worthwhile.

RESULTS AND DISCUSSION

The bacteriologic results are summarized in figure 1 and 2, which include the results of comparable groups infected with *Br. abortus* and superinfected with *Br. suis* from the earlier experiment (Kelly et al, 1953). The group receiving *Br. melitensis* and *Br. suis* simultaneously is omitted from the figures; both organisms were recovered from all animals in this group. The ease with which superinfection could be imposed on primary brucellosis varied with the species used for the respective infections. *Br. suis* could be readily super-

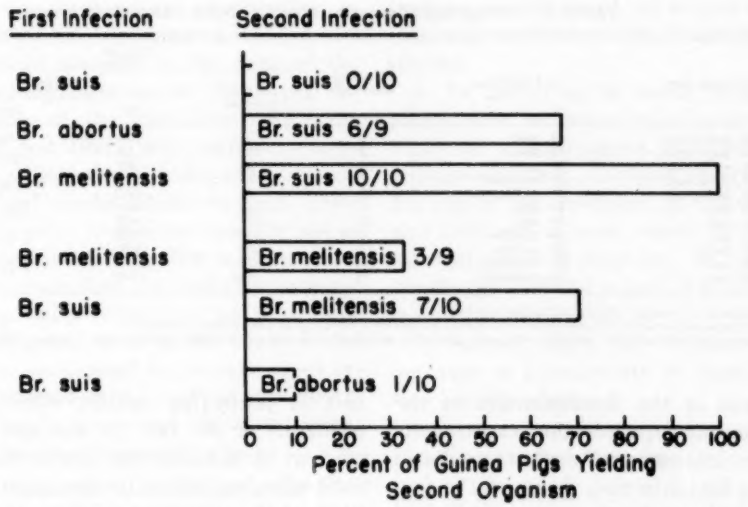

FIG. 2.—Recovery of *Brucella* species superimposed on brucellosis of 15 days duration. The length of the bar indicates the percent of guinea pigs yielding the second organism; the numbers following the name of the second organism represent the number yielding the superinfecting organism over total superinfected.

imposed on *Br. melitensis* infection after 5 or 15 days but less easily on a homologous infection at either interval. *Br. melitensis* could infect guinea pigs infected for 5 days with either *Br. suis* or *Br. melitensis* but was inhibited to some extent by 15-day infections. *Br. abortus* was largely prevented from establishing itself by a *Br. suis* infection of either duration. It may be pointed out, however, that, as shown earlier (Pollack et al, 1952), the disease caused by *Br. abortus* develops slowly and a longer interval between infection and sacrifice might have altered this result.

In this experiment the agglutinin titers (table 2) of the reinfected animals usually slightly exceeded those of either control group. In three of the four groups in which *Br. melitensis* was the primary infecting organism, the reverse was true.

This experiment suggests that specific protective effects may be detected as early as 5 days after the original infection. The "take" of the second organism varied with the combination used. An example may be found in the animals infected with *Br. suis* or *Br. melitensis* 5 days after infection with one or the other of these species. These two organisms, which are of comparable virulence and are closely related antigenically, might be expected to exert similar nonspecific effects when injected in the same dose, yet an infection of 5 days' duration with *Br. suis* protected 3 of 9 animals against superinfection with the same organism, while an infection of the same duration with *Br. melitensis* did not protect against *Br. suis* infection and exerted slight if any protection against reinfection with the homologous organism.

It is pointed out that in studies such as these, the results may not only be attributable to species differences but also to differences between strains within a species. Examination of the results indicates, in any case, that many factors are

TABLE 2.—*Average agglutinin titers of superinfected and control animals.*

1st infection	2nd infection	Infection interval†	Titers*		
			Super-infected	Controls	
				1st	2nd
Br. melitensis	*Br. suis*	0	98	24	64
Br. melitensis	*Br. suis*	5	205	19	128
Br. melitensis	*Br. suis*	15	64	82	
Br. melitensis	*Br. melitensis*	5	39	(19)‡	134
Br. melitensis	*Br. melitensis*	15	63	(82)	
Br. suis	*Br. suis*	5	342	140	178
Br. suis	*Br. suis*	15	432	448	
Br. suis	*Br. abortus*	5	336	(140)	22
Br. suis	*Br. abortus*	15	688	(448)	
Br. suis	*Br. melitensis*	5	472	338	18
Br. suis	*Br. melitensis*	15	416	329	

* Reciprocal of average titer of all animals in group.
† Days between 1st and 2nd infection; all animals sacrificed 4 weeks after the second infection.
‡ Controls for first infection entered with each group for which they served as controls; second entry for each in parentheses.

involved in the determination of the outcome of superinfection experiments besides any nonspecific effects produced by the first infection. Some of the factors which may be operative, as indicated by the results of these experiments, are listed below. It seems probable that most of these factors as well as others would affect the outcome of each combination and the relative influence of each would vary from one combination to the next. The illustrations cited are intended only as examples in which the particular factor under discussion might have dominated.

(a) The antigenic relationship of the two organisms used: This possibly accounts for the effectiveness of *Br. suis* in protecting against homologous reinfection as well as against superinfection with *Br. abortus*, since *Br. suis* and *abortus* are almost indistinguishable serologically. That it is not always the determining factor is indicated by the facts that *Br. melitensis* is less effective than *Br. suis* in protecting against homologous reinfection and that, as shown in previous experiments, *Br. abortus* does not protect against *Br. suis* as well as *Br. suis* does against *Br. abortus*.

(b) The immunogenic potency of the organism used: The strain of *Br. melitensis* used was less effective than *Br. suis* in protecting against either *Br. melitensis* or *Br. suis*. It was also less efficient in inducing the production of antibodies, as judged by the agglutinin titers of the animals receiving only *Br. melitensis*. If the titer of protective antibodies may be assumed to parallel that of agglutinins, it is possible that the greater immunogenicity of *Br. suis* was responsible for its greater effectiveness in protecting against homologous infection.

(c) The characteristics of the disease caused by the two organisms: This may in part account for the difference in results of the *Br. suis* and *Br. abortus* combinations when the order of administration of the organisms was altered; *Br. abortus*, which produces a more slowly progressing infection than does *Br. suis*, was less effective in overcoming a previously established *Br. suis* infection than was *Br. suis* in establishing itself in animals infected with *Br. abortus* (Kelly et al, 1953).

(d) The time elapsing between infections: This was discussed in earlier papers (Pollack et al, 1952; Kelly et al, 1953).

Another factor which might be expected to be of importance and which was not tested in these experiments is the number of organisms injected, especially for the second infection. How-

ever, in exploratory experiments in which the infection interval was 6 weeks a 10-fold increase in the dose of the second organism had no effect upon the outcome of the experiment. McEwen (1940 and 1941), who used very large doses of both first and second organisms, obtained results similar to ours. These results seem to indicate that the size of the second infecting dose may be of less importance than the factors mentioned above. Other factors not tested in these experiments, such as the effect of the strain, as opposed to the species, of organism used or the route of administration of the respective organisms might also have a considerable effect.

SUMMARY AND CONCLUSIONS

1. Guinea pigs were infected with *Brucella suis* or *Brucella melitensis* and superinfected 5 or 15 days later with *Br. suis*, *Br. melitensis*, or *Brucella abortus*. At sacrifice 4 weeks later the presence or absence of the second infection was determined by bacteriologic studies.

2. *Br. suis* could be readily superimposed on *Br. melitensis* infection but less easily on a homologous infection. *Br. melitensis* was superimposed upon either *Br. suis* or *Br. melitensis* at 5 days but was inhibited to some extent by infections of 15 days duration. *Br. abortus* became established in very few animals infected with *Br. suis*.

3. Factors which may influence the outcome of experiments in superinfection are discussed.

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ACTIVITY OF SOME VITAMIN DERIVATIVES AND RELATED COMPOUNDS FOR ORAL LACTOBACILLI

STEWART A. KOSER AND JOAN L. THOMAS

From the Walter G. Zoller Memorial Dental Clinic and the Department of Bacteriology and Parasitology, The University of Chicago, Chicago 37, Illinois

In a previous communication results of a study of vitamin needs of lactobacilli isolated from saliva, plaques, and carious lesions were presented.[1] In the present study some derivatives of required vitamins or compounds with related structures were tested for their ability to replace the vitamin in supporting growth and acid production of oral lactobacilli.

METHODS

Basal medium.—The basal medium used was similar to that previously employed[1] and consisted of 0.5% acid-hydrolyzed purified casein, 1% glucose, 1% sodium acetate·$3H_2O$, and inorganic salts, including 0.2% phosphate; this was supplemented with 100 mg each of L-cystine, L-tryptophane, L-hydroxyproline, DL-threonine and DL-serine, 10 mg each of adenine sulfate, guanine hydrochloride, thymine, hypoxanthine, and uracil per liter of medium. The purines and pyrimidines were added because their presence, along with the needed vitamins, results in speedier growth of many lactobacilli.

The vitamins used for 1000 ml of medium were 10 μg biotin, 1000 μg nicotinic acid, 1000 μg calcium pantothenate, 100 μg pyridoxal hydrochloride, 200 μg riboflavin, 200 μg pteroylglutamic acid, and 100 μg para-aminobenzoic acid. They were added to the medium as it was prepared and were autoclaved in the medium. For heterofermentative lactobacilli from 0.2 to 1.0 μg of thiamine hydrochloride per ml was added separately from a previously filtered solution to individual tubes of medium after they had been autoclaved. In the tests of activity of a vitamin derivative or substitute, the vitamin itself was, of course, omitted from the medium and replaced by the test compound, which was usually added separately after the medium had been tubed and sterilized in the autoclave.

The medium was adjusted to pH 6.8 before autoclaving and was usually about 6.6 after autoclaving. The ingredients for one liter were dissolved in 900 ml of glass redistilled water and the medium placed in 9-ml amounts in tubes standardized for turbidity determinations in the Lumetron. After addition of sterile vitamin solutions or other test substances the total volume was 10 ml per tube.

Vitamin derivatives.—Solutions of the compounds were prepared separately, sterilized by filtration, and added aseptically to the basal medium after it had been sterilized. Each compound was used in several different amounts, and controls with and without the vitamin in question were included in the tests. Some of the compounds are not available commercially and we are indebted to Dr. Klaus Hofmann of the University of Pittsburgh for a sample of *dl*-oxybiotin; to Hoffmann-La Roche Inc. for *d*-norbiotin, *dl*-homobiotin, a second sample of *dl*-oxybiotin, *dl*-desthiobiotin, *d*-biotinol, *d*-panthenol, and 3-pyridine methanol; to Merck and Company for pantolactone and the pyrimidine and thiazole portions of thiamine. Sodium pantoate was prepared from pantolactone by treatment with 0.1 *N* sodium hydroxide according to Stansly and Schlosser.[2] The other compounds used were those available commercially. Quinolinic acid was recrystallized several times.

Cultures.—Fifteen strains of oral lactobacilli, 10 of the homofermentative type and 5 heterofermentative, were used for most of the tests; these strains were from the group of 26 cultures previously used for study of vitamin requirements. All of them need biotin, nicotinic acid, and pantothenic acid for growth; representatives of the homofermentative type need riboflavin, those of the heterofermentative type require thiamine. In addition, many of them either need or are stimulated by pyridoxal (or pyridoxamine), pteroylglutamic acid, or both of these substances.

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None of them needs vitamin B_{12}. In addition, a few named stock laboratory cultures from other sources were included for comparative purposes,—one each of *Lactobacillus casei, arabinosus, fermenti,* and *acidophilus*. In the presentation of results in the following tables the last 4 cultures are included with the 15 oral lactobacilli. All cultures were kept in stock in tomato juice-yeast extract liquid medium, transferring approximately once a month with interim storage in the refrigerator. For inoculation of tests 24-hour cultures were used. These were made from the stock cultures and 0.001 ml, secured by dilution in buffered saline, was used for inoculation of each tube.

Growth and acid production.—Incubation was at 37 C. Response of the lactobacilli to the different compounds was followed by measurements of turbidity of cultures in a Lumetron colorimeter with a 650 mμ filter; also, pH determinations were made in many cases.

RESULTS

Biotin derivatives.—When supplied in place of biotin, some compounds supported good growth of oral lactobacilli, and others showed relatively little growth-promoting activity (table 1). Desthiobiotin in the amount of 0.01 μg per ml did not support growth of any of the cultures, although an equivalent amount of biotin supports good growth. An increase in the amount of desthiobiotin to 1.0 μg per ml produced slow growth of about one-third of the cultures. The pattern of growth differed from that seen in the presence of biotin; growth did not appear until the 3rd to 5th day of incubation, although eventually in many cases it became almost as heavy as that in the biotin control tubes. This is shown in table 2, which gives results for 3 of the 7 cultures capable of growing in the presence of 1.0 μg per ml of desthiobiotin. Evidently desthiobiotin is utilized with difficulty by some oral lactobacilli and not at all by others. These findings with the oral lactobacilli are similar in general to reports dealing with several lactobacilli used for assay purposes; *L. casei, arabinosus,* and *pentosus* do not respond to desthiobiotin in ordinary amounts.[3–6] Some other microorganisms, however, grow readily when desthiobiotin is supplied in place of biotin: a strain of *Leuconostoc destranicum*,[7] some clostridia,[8] *Saccharomyces cerevisiae*,[3,4,9,10] vari-

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TABLE 1.—*Growth-promoting effect of some biotin derivatives.*

Compound	Amount, μg per ml	Composite result for 19 lactobacilli	Activity in relation to biotin on basis of *d*-form
dl-desthiobiotin	0.01 1.0	No growth 7 slow growth, 12 no growth	Less than 0.1%, or 0
dl-oxybiotin	0.001	All good growth, prompt, except one strain	10–100%, often 50–100%
dl-homobiotin	1.25 12.5	5 light or slow growth, 14 no growth 13 moderate to heavy growth, slow; 6 no growth	Less than 0.1%, or 0
d-Norbiotin	1.25	All good growth	About 0.1%
d-Biotinol	0.0001 0.001 0.01 1.0	Growth very light, or none Mostly light, 3 no growth Moderate growth, 3 no growth Mostly good growth, prompt; 1 slow, 1 negative	0.1% or less

TABLE 2.—*Comparative growth-promoting effect of desthiobiotin and biotin.*

Culture no.	Test medium* plus	Amount, μg per ml	Growth shown by turbidity† Days			
			2	4	6	9
1009	No biotin		3	3	4	6
	d-biotin	0.005	78	80	84	85
	dl-desthiobiotin	0.01	5	6	8	9
	dl-desthiobiotin	1.0	2	4	48	75
1010	No biotin		3	4	5	6
	d-biotin	0.005	79	83	84	85
	dl-desthiobiotin	0.01	3	4	6	7
	dl-desthiobiotin	1.0	2	4	60	76
1011	No biotin		4	9	10	12
	d-biotin	0.005	75	76	80	82
	dl-desthiobiotin	0.01	7	11	13	14
	dl-desthiobiotin	1.0	3	21	39	44

* The test medium contained all vitamins needed by these cultures except biotin.

† Increasing turbidities are shown by progressively higher figures. 0 = a clear tube, similar to the uninoculated medium; values of about 60 to 80 are very turbid cultures, i.e. heavy turbidity by ordinary visual inspection.

ous other yeasts and some filamentous fungi.[5]

In contrast to the foregoing results, *dl*-oxybiotin in concentration of 0.001 μg per ml supported good growth. A more detailed examination of the comparative effect of oxybiotin and biotin, using suboptimum amounts of these vitamins, showed that in a few cases oxybiotin was somewhat less active than biotin. In such cases, growth started somewhat later in the presence of small amounts of oxybiotin and did not always attain the luxuriance reached when equivalent amounts of biotin were supplied. The difference was apparent after allowing for the assumption that only the *d*-form of *dl*-oxybiotin is active. However, in tests with a number of other lactobacilli, the responses to graded amounts of oxybiotin were more nearly equal to those produced by an equivalent amount of biotin.

All of our tests were made in a casein digest basal medium, which contained about 200 μg per ml of aspartic acid, based on the amino acid content of casein. Previous reports have indicated that under these conditions the activity of oxybiotin is relatively high and may approach or equal that of biotin for the several strains of lactobacilli used for assay purposes.[6,11,12] Oxybiotin has been reported to show a relatively high order of activity for *Rhizobium trifolii*, *Saccharomyces cerevisiae*,[11,12] and *Leuconostoc dextranicum*.[7] For several species of *Clostridium* the activity of *dl*-oxybiotin ranged for the most part from 15 to 60% of that of biotin, but in a few cases was as low as 0 to 5%.[8] Our results with the oral lactobacilli are quite similar to those reported for the assay strains of *L. arabinosus* and *L. casei*. We did not include any tests of relative activity in the absence of aspartic acid, but it has been shown that under such conditions the activity of oxybiotin for *L. arabinosus* and *L. casei* is much lower in relation to that of biotin,[13] particularly at an initial pH of about 6.5.[14] All of the lactobacilli used in these tests required biotin for growth in the casein digest medium. Thus, for these organisms aspartic acid does not substitute for biotin as effectively as, for example, in the case of *Torula cremoris*.[15]

Norbiotin and homobiotin are homologs of biotin differing in the length of the side chain. The $(CH^2)^4$ chain of biotin is replaced by $(CH^2)^3$ in the case of norbiotin and by $(CH^2)^5$ in homobiotin. Homobiotin exhibited little growth-promoting activity when supplied in ordinary amounts; 1.25 μg per ml supported light and slow growth only in some cases. An increase in the amount

11. Winnick, T., Hofmann, K., Pilgrim, E. J. and Axelrod, A. E. 1945, J. Biol. Chem. **161**: 405–410.
12. Rubin, S. H., Flower, D., Rosen, F. and Drekter, L. 1945, Arch. Biochem. **8**: 79–90.
13. Stokes, J. L., Larsen, A. and Gunness, M. 1947, J. Bact. **54**: 219–230.
14. Potter, R. L. and Elvehjem, C. A. 1950, J. Biol. Chem. **183**: 587–592.
15. Koser, S. A., Wright, M. H. and Dorfman, A. 1942, Proc. Soc. Exper. Biol. & Med. **51**: 204–205.

of 12.5 μg per ml produced a more evident response, though again growth was slow, often 3 to 7 days being necessary for the attainment of moderate to heavy growth, in contrast to the prompt response to biotin.

Norbiotin, 1.25 μg per ml, supported good growth of most of the lactobacilli and growth occurred about as promptly as in the presence of biotin. But when smaller amounts of norbiotin were supplied, it was clear the activity of this compound is of a much lower order than that of biotin. This is seen in table 3, which gives comparative results with 2 lactobacilli. One-tenth to 1.0 μg of norbiotin was required for heavy growth in contrast to the 0.0001 to 0.0002 μg amounts of biotin. Results similar to those of table 3 were obtained with 2 other strains of oral lactobacilli. Since norbiotin possessed about one-thousandth the activity of biotin, the question arises of the possibility of traces of biotin in the norbiotin. Belcher and Lichstein,[16] on testing the response of yeasts to norbiotin and homobiotin, believed that differences in the pattern of growth were indicative of lack of contamination with biotin. From our results with norbiotin and the lactobacilli it is impossible to draw a definite conclusion concerning this point.

16. Belcher, M. R. and Lichstein, H. C. 1949, J. Bact. **58:** 579–583.

TABLE 3.—*Comparative activity of norbiotin and biotin.*

Vitamin	Amount, μg per ml	*Lactobacillus* 46 Growth at hours: 24	44	68	*Lactobacillus* 1009 Growth at hours: 24	44	68
d-norbiotin	0		2	5	6	11	13
	0.001	3	3	7	4	10	13
	0.01	7	22	28	16	25	29
	0.1	20	67	72	35	70	72
	1.0	23	84	87	32	82	84
d-biotin	0.00001	7	20	27	13	22	24
	0.00005	23	53	56	32	50	51
	0.0001	22	64	67	24	65	65
	0.0002	26	75	81	29	76	78

TABLE 4.—*Growth-promoting effect of biotinol compared with that of biotin.*

Culture	Biotinol, μg per ml	Growth at days: 1	2	4	7
46	0.001*		3	8	9
	0.01	0	19		43
	1.0	22	84	86	
	0.001 biotin	25	84	87	
1008†	0.001	10	23		16
	1.0	20	79		75
	0.001 biotin	56	82		80
L. casei	0.001	4	12		17
	0.01	12	51	60	64
	1.0	27	85	89	89
	0.001 biotin	24	84	88	

* The amounts shown are for biotinol except where biotin is specified.
† Heterofermentative type.

The alcohol analog of biotin, *d*-biotinol, was at most not more than 0.1% as active as biotin (table 1). A more detailed comparison of the response of a few of the lactobacilli to these 2 compounds is given in table 4. Our results are, in general, similar to those of Drekter and associates,[17] who found the activity of biotinol, after purification, to be less than 0.05% that of biotin for *L. arabinosus*, *L. casei*, and *S. cerevisiae*. It is of interest that biotinol is reported to possess a high order of activity, approaching that of biotin, for the rat and man.[17]

Compounds related to pantothenic acid.—Sodium pantoate, β-alanine, and the pantolactone moiety of pantothenic acid were tested separately and in several combinations in amounts up to a total of 10 μg per ml. All of the lactobacilli used for these tests require pantothenic acid for growth, but none of them grew when the above compounds were supplied as substitutes. These results with oral lactobacilli are similar to those reported for the same compounds in the case of *Lactobacillus*

17. Drekter, K., Scheiner, J., DeRitter, E. and Rubin, S. H. 1951, Proc. Soc. Exper. Biol. & Med. **78:** 381–383.

bulgaricus and *helveticus*,[18] and for various lactobacilli, streptococci, and *Leuconostoc* species.[19]

d-Panthenol, the alcohol analog of pantothenic acid, was used in amounts of 1.0 and 50 μg per ml, but failed uniformly to support growth of any of the lactobacilli when supplied in place of pantothenic acid. These negative results are in line with the report of Snell and Shive,[20] who, in a study of growth inhibition by analogs of pantothenic acid, noted that panthenol did not replace pantothenic acid for growth of *S. cerevisiae*, 2 streptococci, several lactobacilli, and *Leuconostoc mesenteroides*. In some cases panthenol inhibited growth induced by pantothenic acid.

Compounds related to nicotinic acid.—The growth-promoting effect of some nicotinic acid derivatives and related compounds is summarized in table 5. The isomers of nicotinic acid, isonicotinic acid and picolinic acid were inactive in ordinary vitamin amounts, such as 1.0 μg per ml, but isonicotinic acid showed a slight growth-promoting effect at 20 μg per ml. Whether this effect is due to the compound itself or to traces of active substances as impurities is not known. The two isomers in previous tests[21–23] with other microorganisms exhibited no growth-promoting effect in amounts up to $10^{-4}M$, or about 12 μg per ml.

Results with the remaining compounds are similar for the most part to

18. McRorie, R. A. and Williams, W. L. 1951, J. Bact. **61**: 737–745.
19. Cheldelin, V. H., Hoag, E. H. and Sarett, H. P. 1945, J. Bact. **49**: 41–45.
20. Snell, E. E. and Shive, W. 1945, J. Biol. Chem. **158**: 551–559.
21. Knight, B. C. J. G. and McIlwain, H. 1938, Biochem. J. **32**: 1241–1251; Landy M. 1939, Proc. Soc. Exper. Biol. & Med. **38**: 504–506.
22. Lwoff, A. and Querido, A. 1939, Compt. rend. Soc. de biol. **130**: 1569; Pelczar, M. J., Jr. and Porter, J. R. 1940, J. Bact. **39**: 429–435; Ellinger, P., Fraenkel, G. and Abdel Kader, M. M. 1947, Biochem. J. **41**: 559–568.
23. Dorfman, A., Koser, S. A., Reames, H. R., Swingle, K. F. and Saunders, F. 1939, J. Infect. Dis. **65**: 163–182.

TABLE 5.—*Growth-promoting effect of nicotinic acid derivatives and some related compounds.*

Compound	Amounts tested, μg per ml	Composite result for 19 lactobacilli
Isonicotinic acid	1	No growth
	20	Light growth
Picolinic acid	1	No growth
	20	No growth
Quinolinic acid	1	No growth
	5	No growth
	20	Light or no growth
Quinolinic diamide	20	No growth
6-Methyl nicotinic acid	20	No growth
2-Amino nicotinic acid	1	No growth
	100	No growth
Nicotinyl monoethylamide	1	Mostly fair to good growth, some light or no growth
	10	Mostly good growth, few slow or no growth
Nicotinyl diethylamide	1	Light or no growth
	10	Fair to good growth, few no growth
3-Pyridine methanol	1	No growth
	50	Moderate or good growth of some, others poor or no growth
3-Hydroxy anthranilic acid	1	No growth
	10	No growth
Pyrazine monocarboxylic acid	20	No growth
Pyrazine dicarboxylic acid	20	No growth

those reported for other nicotinic acid-requiring microorganisms. Quinolinic acid supported some growth at 20 μg but not at 5 μg per ml. This compound has been reported variously either as exhibiting some growth-promoting activity in similar concentrations or at times as inactive for several different microorganisms: *Staphylococcus aureus*,[21] *Proteus*,[22] and dysentery bacilli.[23] There is the possibility that the activity observed in the higher concentrations may be due to the presence of small amounts of nicotinic acid. Concerning the mono- and diethylamides of nicotinamide, the former was somewhat more active than the latter though various strains of lactobaccilli responded somewhat differently. 3-Pyridine methanol was devoid of activity in ordinary amount, but showed some growth-promoting effect in higher concentration. Since some cultures failed to respond at the higher level it seems probable the growth-promoting effect was not due to small amounts of nicotinic acid or other active substances as impurity in the preparation. 3-Hydroxy anthranilic acid, which is believed to be an intermediate in the conversion of tryptophane to nicotinic acid by *Neurospora*,[24] showed no growth-promoting activity for any of the lactobacilli. Evidently the lactobacilli are unable to bring about the conversion to nicotinic acid. Similar results for *L. arabinosus* 17-5 and several other bacteria used for assay purposes have been reported by Volcani and Snell.[25] The two pyrazine compounds, which contain two nitrogens in the ring structure, were inactive as growth-promoting agents.

Components of thiamine.—Heterofermentative lactobacilli, in contrast to those of the homofermentative type, require thiamine for growth. The results of tests in which the pyrimidine and thiazole components were substituted for thiamine are given in table 6. The pyrimidine did not support growth, but the thiazole component supported growth of the majority of the cultures. With the pyrimidine and thiazole together there was at times a somewhat more prompt growth response; most of the cultures attained heavy turbidity within 24 or 48 hours following a small inoculation. Two strains failed to grow when 0.1 μg per ml of each of the components was supplied, but grew when the amount was increased 100-fold to 10 μg per ml. The several tests with the pyrimidine and thiazole components of thiamine were repeated, using a basal medium lacking the other pyrimidines and purines usually supplied in the medium, but the results were not essentially different under these conditions. Evidently these organisms do not require the intact thiamine molecule; most of them respond to the thiazole portion alone or to the thiazole and

TABLE 6.—*The pyrimidine and thiazole components of thiamine as growth-promoting agents for heterofermentative lactobacilli.*

Compound	Amount, μg per ml	Composite result for 10 strains*
Pyrimidine	0.5	No growth
Pyrimidine	10.0	No growth
Thiazole	0.5	8 good growth 2 no growth
Thiazole	10.0	8 good growth 2 no growth
Pyrimidine + thiazole	0.1 each	8 good growth 2 no growth
Pyrimidine + thiazole	10.0 each	10 good growth†
Thiamine (control)	0.2	10 good growth†

* Some additional heterofermentative lactobacilli of oral origin were used for this test, in addition to the 5 strains used in previous tests.

† Most of the 10 cultures responded somewhat more promptly to thiamine than to the pyrimidine plus thiazole combination or thiazole alone; eventually; however, about the same turbidity and pH levels were reached.

24. Beadle, G. W., Mitchell, H. K. and Nyc, J. F. 1947, Proc. Nat. Acad. Sc. **33**: 155–158; Mitchell, H. K. and Nyc, J. F. 1948, Proc. Nat. Acad. Sc. **34**: 1–5.

25. Volcani, B. E. and Snell, E. E. 1948, Proc. Soc. Exper. Biol. & Med. **67**: 511–513.

TABLE 7.—*Hydrogen ion concentration attained in the presence of some vitamin derivatives.*

Compound	Amount, μg per ml	Number of cultures and fermentative type	pH range	Turbidity
dl-oxybiotin	0.002	12 homo	4.00–4.07	80–88
	0.002	5 hetero	4.53–4.59	68–85
d-norbiotin	1.0	12 homo	4.00–4.16	70–85
	1.0	5 hetero	4.60–4.73	66–80
Thiazole plus pyrimidine components of thiamine	0.1 each	8 hetero	4.50–4.59*	63–80*

* Growth was slower than in the presence of an equivalent amount of thiamine but later (48 to 96 hours) comparable turbidities and pH values were attained.

pyrimidine in ordinary amounts. The response, however, is often somewhat slower than to thiamine. In the extensive literature on lactobacilli there seems to be little information on the effectiveness of the two components of thiamine. Sarett and Cheldelin,[26] when proposing the use of *L. fermenti* 36 (ATCC 9338) for assay of thiamine, noted that this organism responded to pyrimidine plus thiazole somewhat more slowly than to thiamine, requiring 40 to 48 hours in the former case, compared to 18 hours in the presence of thiamine.

pH.—Determinations of final hydrogen ion concentration of cultures were made in those instances where a vitamin derivative supported growth. Where the amount of growth, as measured by turbidity, was about the same as that supported by an optimal amount of the vitamin itself, the resultant lowering of pH in the presence of the vitamin derivative was usually comparable to that attained with the vitamin. A few typical examples are given in table 7. In each case the pH values reached in the presence of adequate amounts of oxybiotin, norbiotin, or the pyrimidine and thiazole components of thiamine are comparable to those reached by the same cultures in the presence of the respective vitamins (not shown in the table) in the basal medium employed for these tests. In other instances where less than optimal growth appeared in the presence of a vitamin derivative, lowering of the pH value bore about the same general relationship to turbidity as that found in the presence of suboptimal amounts of the vitamin. In no instance did a vitamin derivative support good growth of the lactobacilli with, at the same time, little acid production.

26. Sarett, H. P. and Cheldelin, V. H. 1944, J. Biol. Chem. **155**: 153–160.

SUMMARY

Nineteen strains of lactobacilli, 15 of oral origin and 4 named stock laboratory cultures, were subjected to tests of growth response in a casein digest medium when a vitamin derivative or other related compound was substituted for a required vitamin. Of the biotin substitutes, only oxybiotin supported prompt and vigorous growth when supplied in small amounts, such as 0.001 μg per ml. Desthiobiotin at 1.0 μg per ml elicited a poor response on the part of some cultures, and no response from others. Norbiotin was about one-thousandth as active as biotin but supported good growth when used in amounts of 0.1 and 1.0 μg per ml. Homobiotin was distinctly less active than norbiotin. Biotinol possessed only about 0.1% or less the activity of biotin.

Of nicotinic acid derivatives, isonicotinic acid, picolinic acid and quinolinic acid were inactive or essentially inactive. Nicotinyl monoethylamide, supported moderately good growth of most strains; the corresponding diethyl-

amide was less active. 3-Pyridine methanol supported moderately good growth of some cultures only at a level considerably above that usually used for nicotinic acid. The possibility of contamination of some of these compounds with traces of nicotinic acid or related active substances cannot be definitely excluded, so that a moderate or slight growth in the presence of a large amount of the substance does not necessarily indicate low grade utilization of the compound. 2-Amino nicotinic acid, 6-methyl nicotinic acid, quinolinic diamide, 3-hydroxy anthranilic acid, and two pyrazine carboxylic acids were inactive as growth-promoting agents at any concentration tested.

Sodium pantoate, β-alanine, and pantolactone, separately and in combination in various amounts, uniformly failed to support growth when supplied as substitutes for pantothenic acid.

The thiazole component of thiamine substituted fairly satisfactorily for thiamine in promoting growth of most heterofermentative lactobacilli. Growth did not occur as promptly as in the presence of thiamine but eventually attained comparable luxuriance.

EXPERIMENTAL INFECTIONS OF RHESUS MONKEYS WITH *SCHISTOSOMATIUM DOUTHITTI* (CORT, 1914)

IRVING G. KAGAN

From the Zoological Laboratory, University of Pennsylvania, Philadelphia 4, Pennsylvania

In the United States bathers are occasionally exposed to the cercariae of several bird and at least one mammalian schistosome. Penetration into the skin by these cercariae may, in a sensitized individual, produce a schistosome dermatitis. The fate of these cercariae in man is unknown although it is currently believed from biopsies of skin tissue that cercariae do not penetrate beyond the epidermal layers of the skin in humans (Brackett, 1940a). Oliver (1949a) exposed laboratory animals, including a rhesus monkey, to several species of bird schistosomes and observed schistosome lesions in the lungs of these animals. Obviously the cercariae of dermatitis-producing species are not all immobilized in the skin of mammalian hosts.

The question of whether animal schistosomes are infective for man has been raised repeatedly in the literature. Penner (1941) exposed a young rhesus monkey to 28,000 cercariae of *Schistosomatium douthitti*, a dermatitis-producing mouse species endemic in the United States, and recovered young schistosomulae in the lungs 5½ days after exposure. This experiment suggests that primates may be susceptible to systemic infections with animal schistosomes.

In a series of experiments reported in this paper this hypothesis was critically tested. The development of *S. douthitti* was studied in rhesus monkeys, *Macaca mulatta*, exposed to large numbers of cercariae and autopsied at closely spaced intervals over a period of several weeks. Resistance experiments with monkeys exposed to *S. douthitti* against the homologous species and against *Schistosoma mansoni* were also conducted.

MATERIALS AND METHODS

Four young rhesus monkeys (#1, 6, 10, 13) approximately 2 to 4 years of age were obtained from a dealer. The other 12 monkeys were previously used in virus and toxin studies. All animals were healthy and active when brought into the laboratory and were maintained on a stock diet of a prepared mash, vegetables and fruit.

Cercariae from snails, *Lymnaea palustris*, reared and infected in the laboratory or collected from small ponds in the vicinity of Chicago, Illinois, were used in this study. When infected snails are isolated during the day, cercariae appear in the water several hours after dark, swim about for a brief period and then rise to the surface film of the water, where they remain motionless. To insure that viable cercariae were used, exposure was always made at night. The monkey was anesthetized with an interperitoneal injection of 1 to 1½ cc of veterinary sodium pentobarbital and the abdominal hair removed with animal clippers. Cercariae were lifted from the surface film of the containers, holding infected snails with a small wire loop, the number roughly estimated under a binocular microscope and then placed directly on the moistened skin. On two occasions mice were also exposed as controls.

Prior to necropsy the animal was again anesthetized, bled from the heart to obtain serum for in vitro studies, and injected with 8 to 10 mg of heparin to prevent clotting. Animals were perfused with a 2% citrate-0.85% saline mixture (Yolles et al, 1947). The liver was litigated, re-

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moved from the body and perfused separately. The intestinal veins and lungs were perfused from the dorsal aorta. Young worms recovered at necropsy were fixed in cold Bouin's and stained with Mayer's hydrochloric carmine. Small pieces of lung, liver, spleen and lymph nodes were fixed in Zenker-10% formal mixture for histological examination.

In resistance experiments the monkey to be tested and a control monkey were exposed to cercariae from the same snails. The criteria for determining the presence or absence of immunity were the number of worms recovered in the control as compared to the test animal, weight and behavior following exposure and pathological changes in the lungs at necropsy.

TABLE 1.—*Recovery of* S. douthitti *in rhesus monkeys.*

No.	Weight in g. and sex	Number of cercariae	Days between exposure and necropsy	Number of worms recovered	Location*
1	2000 (F)	2,500	10	30†	L
2	2750 (M)	45,000	11	500	L, Lu
3	2550 (M)	7,000	8–12	3	L
4	2770 (M)	7,500	11–14	2	L
5	2700 (M)	10,000	15	1	L
6	2300 (F)	5,000	16	2	L
7	1970 (F)	7,000	19	1,000	L, M
8	1600 (F)	6,000	22–23	200	L, M
9	2725 (F)	10,000	24–25	8	L, M
10	3750 (M)	6,000	30	Neg.	
11	2525 (F)	7,000	35	Neg.	

* L = liver; Lu = lungs; M = mesenteric veins.
† A portion of the perfusate with young schistisomulae was inadvertently lost.

EXPERIMENTAL RESULTS

Sexually mature or young *S. douthitti* were recovered in the venules of the liver and intestinal wall of 9 rhesus monkeys exposed to 2,500 to 45,000 cercariae of *S. douthitti*. The number of parasites recovered at necropsy varied from 1 to 1000 worms (table 1). In all but 4 infections, the schistosomulae at necropsy appeared to be growing normally. Comparison of schistosomes of similar age recovered in mice with worms in monkeys revealed that in the monkey the schistotomes were smaller and not as well developed. In the venules of the liver of 4 animals necropsied 8 to 16 days after exposure small schistosomulae were recovered similar to worms in mice of 4 to 5 days development.

Five monkeys necropsied 30 to 96 days after exposure were negative for schistosomes. Cercariae that had been used to expose one of these negative animals were infective for mice. Dead and dying worms were recovered in monkey #8, exposed on two successive evenings 22 and 23 days prior to necropsy. Although approximately half of the 200 worms recovered in this animal were normal, 6 dead sexually mature female worms were recovered and the remaining worms were atypical in appearance. One or two dead worms were recovered in monkeys #4 and #6 necropsied 14 and 16 days after exposure. The recovery of dead worms or 1 to 3 living worms at necropsy indicates that resistance to infection may begin as early as the second week following infection. The absence of worms in 5 animals necropsied after 30 to 92 days suggests that the infection is completely terminated in approximately 3 to 4 weeks.

Without exception all 16 monkeys exposed for the first time to cercariae of *S. douthitti* developed a schistosome dermatitis. Within ½ to 1 hour after exposure small macules appeared at the cite of cercariae penetration, and occasionally papules were formed. In approximately 24 hours a diffuse erythema was noted in the area of cercariae penetration, which increased in intensity for another 24 hours. The erythema gradually diminished and faded away by the 5th to the 7th day. Animals were observed scratching the exposed areas of skin. In four experiments monkeys were re-exposed to cercariae 34 to 82 days after initial exposure. Animals that were never previously exposed were used as controls. The dermatitis which developed in the controls was always more intense than the dermatitis produced in the animal being exposed for a second time. One monkey, exposed for a second time, developed white wheals without

erythema, whereas the control developed a typical erythema. Two monkeys (#2, 5) that had been exposed to approximately 10,000 cercariae showed evidence of distress and discomfort several days after exposure. Appetite fell off until food was completely rejected. These monkeys crouched in the corner of their cage with their heads between their feet and appeared to be ill. Recovery began the 8th to 10th day subsequent to exposure and both appetite and activity soon returned to normal. At necropsy these animals show signs of hemorrhage and damage in the lungs. Except for the lungs, the internal organs appeared to be normal.

Resistance against a homologous second exposure to cercariae of *S. douthitti* was tested in three monkeys (table 2). Although no conclusion can be drawn on the basis of the number of worms recovered from the challenging exposure in control animals, there is evidence that in animals previously exposed cercariae from the challenging infection die before they reach the lungs. In addition, the host exposed to a second infection does not show the loss of weight and other clinical symptoms characteristic of a first infection. The duration of resistance in previously exposed animals is at least 82 days (end of test period).

In test B (table 2) one worm was recovered in a control animal (#5) and no worms in the re-exposed monkey (#13). The control animal lost 300 g of weight between exposure and necropsy, whereas the previously infected monkey (#13) showed no discomfort and gained 30 g of weight. In the test monkey a slight amount of erythema was observed the following day in the area of cercarial penetration. In the control monkey a flaming erythema developed in 24 hours. At necropsy the lungs of the control showed evidence of much hemorrhage, whereas the lungs of the immune animal were normal.

In test C (table 2) three worms were recovered in the control and no worms in the test animal. The differences observed in the dermatitis produced at exposure were very striking. These two animals were exposed by placing them side by side and alternating applying cercariae to their moistened skins. Shortly after exposing the immune animal a blanching of the skin in the area of cercarial penetration was noted, followed by the formation of papules. Twenty-four hours later this animal did not develop any erythema in the exposed

TABLE 2.—*Immunity to re-exposure with cercariae of* S. douthitti *or* S. mansoni *after a primary exposure to the cercariae of* S. douthitti.

Test	No. of monkey	Weigh in g. and sex	Cercariae of *S. douthitti* in first exposure	Days between exposures	Cercariae of *S. douthitti* in second exposure	Days between 2nd exposure and necropsy	Number of worms recovered
A	12	3485 (M)	8,000	35	7,500	10	0
	4	2770 (M)			7,500	11	2
B	13	2400 (F)	12,000	40	10,000	15	0
	5	2700 (M)			10,000	15	1
C	14	2460 (M)	10,000	82	7,000	8–12	0
	3	2550 (M)			7,000	8–12	3
D	15	2375 (F)	8,000	34	700 (*S. mansoni*)	58	28
				81	6,000	15	0
	16	3970 (M)			700 (*S. mansoni*)	58	18
				44*	6,000	14	0

* Days following exposure to *S. mansoni* infection.

area. Exposure of the control animal produced no apparent blanching of the skin or papule formation; however, in 24 hours the area was very erythematous. At necropsy the lungs of the immune animal were normal, whereas the lungs of the control showed signs of hemorrhage. Shortly after exposure the control became ill and in 4 days lost 300 g of weight, while the immune animal showed no signs of discomfort and did not lose weight.

A previous infection of *S. douthitti* does not protect a monkey from infection with *S. mansoni*, as demonstrated in test D. Monkey #15 was exposed to *S. douthitti* and 34 days later was reexposed to *S. mansoni* by immersion of the tail in a test tube containing 700 cercariae. Control animal, #16, received 700 cercariae from the same lot of snails in a similar manner. Forty-eight days after exposure to *S. mansoni* both animals were exposed again to 6000 cercariae of *S. douthitti*. The dermatitis produced by the exposure of *S. douthitti* cercariae for the second time in monkey #15 was not as intense as the dermatitis produced in monkey #16. At autopsy 15 days later both animals were positive for the mansoni infection and negative for the douthitti infection.

DISCUSSION

The family Schistosomatidae is characterized, with few exceptions, by a narrow host range. The factors responsible for natural resistance to animal parasites are obscure. Animals exposed to schistosomiasis demonstrate varying degrees of host resistance. The mouse, unlike the rat, is a good laboratory host for *S. mansoni*. The rat develops resistance approximately 6 weeks after infection, which terminates in a self-cure (Stirewalt, Kuntz and Evans, 1951). Fairly (1927) discovered that *Schistosoma spindalis*, a parasite of water buffalo and goats, developed in monkeys for only 11 to 15 days. Monkeys exposed to *S. douthitti*, in the present experiment, were susceptible to infections for approximately 3 to 4 weeks. The mechanism for the rapid death and destruction of the schistosomes of both *S. doubhitti* and *S. spindalis* in the systemic system of the monkey is unknown. Fairly (1927) was able to demonstrate complement-fixing antibodies in his infected animals. Studies in progress with the serums of monkeys infected with *S. douthitti* indicate that circulating antibodies may be detected after infection.

Schistosome dermatitis is generally supposed to be a sensitization phenomenon because the inflammatory response to invading cercariae increases in susceptible hosts with each succeeding exposure (Oliver, 1949b). The schistosome dermatitis observed in monkeys, however, does not appear to follow this pattern, for animals exposed a second time do not develop as intense a reaction as after an initial exposure. The host's response in this experiment may be due to the fact that the monkeys were exposed to thousands of cercariae in the second exposure, which may have desensitized them.

Schistosome dermatitis is regarded by Cort (1950) as a defense mechanism on the part of the host against alien cercariae. In spite of a very severe dermatitis which appeared after initial exposure to cercariae of *S. douthitti*, monkeys became infected—in one instance quite heavily. From the pathology of the lungs in exposed animals it is evident that many cercariae reach the lungs after breaching the skin barrier.

Two of the nine monkeys infected with *S. douthitti* were obtained from a dealer. A third animal purchased was negative 30 days after exposure and a fourth animal was used in a resistance

experiment. Results obtained with these animals were similar to those obtained with the other animals in this study. I do not believe that the previous laboratory history of the discarded monkeys had any influence on the schistosome infections.

It is interesting to note that with one exception (monkey #2) infections in female animals weighing 2000 g or less were larger than infections in male animals. Although the sample is too small for statistical analyses, the influence of sex and age of the host as factors in determining susceptibility to infection should not be completely overlooked.

The site of cercariae immobilization in resistant animals is unknown. From the pathological changes in the lungs and the ease with which animals previously infected withstood massive reexposures to thousands of cercariae, one may assume that in resistant monkeys cercariae of *S. douthitti* are probably immobilized before they reach the lungs.

At least 8 species of monkeys have been experimentally infected with various schistosomes (table 3). Natural infections of *Schistosoma hematobium* (?) by Cobbold (1859) and *S. mansoni* by Cameron (1928) have also been reported from monkeys. Monkeys are probably not equally susceptible to infections with *S. mansoni* and *Schistosoma japonicum*. For example, after 4½ years, Lui and Bang (1950) were able to recover eggs of *S. japonicum* in feces of animals exposed initially to a light infection. Cram and Files (1947) and Standen (1949) reported that some monkeys exposed to *S. mansoni* cease to pass eggs in their feces as early as 4 months after exposure.

The duration and infectivity of animal schistosomes for monkeys varies. Brumpt (1936) found that *Schistosoma bovis*, a bovine species, did not completely develop in *Macacus sinicus* but did reach sexual maturity in *Macacus rhesus*. Liston and Soparkar (1918) were unable to infect *Macacus rhesus* with *S. spindalis*. Fairley (1927), how-

TABLE 3.—*Species of monkeys reported as hosts for schistosomiasis.*

Species	Schistosome	Author
1. *Cercocebus fuliginosus*	*Bilharzia magna* (*S. hematobium?*)	Cobbolt (1859)
2. *Cercocebus* sp.	*S. mansoni* *S. hematobium*	Lieper (1915)
3. *Cercopithecus aethiops*	*S. mansoni* *S. hematobium*	Fairley (1920)
*4. *Cercopitheus sabaeus*	*S. mansoni*	Cameron (1928)
*5. *Macacus cynamolagus*	*S. japonicum*	Africa & Garcia (1941)
6. *Macacus philippinensis*	*S. japonicum*	Lui and Bang (1950)
7. *Macacus mulatta*	*S. mansoni*	Standen (1949) Cram and Files (1947)
	S. hematobium	Standen (1949) Cram and Files (1947)
	S. douthitti	Penner (1941)
8. *Macacus sinicus*	*S. spindalis*	Fairley (1927) Fairley, Mackei and Jasudasan (1930)
9. *Macacus rhesus*	*S. bovis* *S. japonicum*	Brumpt (1936) Vogel (1949)

* Since submitting this paper for publication additional references have been found. *Macacus cynamolagus* has been experimentally infected with *S. hematobium* (Brumpt, E. 1928, Réalisation expérimentale du cycle complet du *Schistosoma hematobium*. Ann. Par. Hum. Comp. **6**: 440–446); *Cercopitheus sabeus* with *S. hematobium* (Archibold, R. G. 1923, Some investigations connected with the spread of bilharziasis in the Dongola Province of the Sudan. Tr. Roy. Soc. Trop. Med. & Hyg. **16**: 419–426); *Cercopithecus pygerythrus* with *S. mansoni* and *S. mettheei* (Blackie, W. K. 1932, A helminthological survey of southern Rhodesia, Mem. Lon. Sch. Hyg. & Trop. Med. **5**: 1–91). A natural infection in a baboon, *Papio porcarius*, with *S. mettheei*, was also reported by Blackie (1932).

ever, was able to infect *M. sinicus* with this species although the duration of infection was only 11 to 15 days.

The short duration of infection of *S. douthitti* in monkeys may explain the negative reports of other workers who exposed these animals to this species. Brackett (1940b) exposed a young rhesus monkey to large numbers of cercariae over a period of several days. Since he autopsied this animal 4 weeks after the last exposure he found it was negative. Penner (1941) exposed two animals. One was necropsied 6 weeks after initial exposure and found to be negative, whereas the second animal necropsied 5½ days after exposure was positive for young schistosomulae in the lungs.

Since *S. douthitti* may develop to sexual maturity in the rhesus monkey, one wonders if it may also develop in man. Although the cercariae of bird schistotomes, *Cercaria stagnicoloe, elvae,* and *physellae,* are the common dermatitis cercariae in the United States, the cercariae of *S. douthitti* have been reported in surveys of Michigan lakes and beaches (McMullen and Brachett, 1948). Raper (1951) briefly reviewed the literature pertaining to the susceptibility of man to animal schistosomes and reported a systemic infection in a medical officer returning to England from Africa with *S. bovis,* a schistosome of cattle. Two days after exposure this individual observed a dermatitis on his body. There were practically no symptoms and he found typical bovis eggs only twice in his feces. The infection would have passed unnoticed in anyone but a trained person.

The transient nature of both *S. douthitti* and *S. spindalis* infections in monkeys and the report of *S. bovis* in man implies that man may not be absolutely resistant towards animal schistosomiasis. Both *S. douthitti* and *S. bovis* reached sexual maturity (*S. spindalis* may not) before the host eradicates the worms. This may be important because it was suggested from previous studies (Kagan 1952a and b, 1953) that the egg of *S. douthitti* may be the antigenic stimulus in the development of acquired immunity against *S. douthitti* in the mouse.

SUMMARY

Sexually mature or young developing male and female worms of *Schistosomatium douthitti* were recovered from the venules of the liver or intestinal veins of 9 rhesus monkeys (*Macaca mulatta*) exposed to the cercariae of *S. douthitti.* These animals were exposed to 2500 to 45,000 cercariae and necropsied 10 to 25 days later. Five monkeys exposed to 6000 to 10,000 cercariae of *S. douthitti* and necropsied 30 to 96 days after exposure were negative.

The recovery of dead and dying worms from monkeys necropsied 10 to 25 days after exposure suggests that resistance to infection in this host may begin as early as the second week after exposure. The recovery of no worms in 5 animals necropsied 30 to 96 days after exposure suggests that the infection is completely terminated in 3 to 4 weeks.

Monkeys exposed to the cercariae of *S. douthitti* develop a schistosome dermatitis very similar to the dermatitis reported for man. In the monkey the rash disappears in 5 to 7 days. In animals previously exposed to the cercariae of *S. douthitti* the dermatitis which develops after second exposure was not as intense as the dermatitis which developed in animals exposed for the first time.

Monkeys exposed to cercariae of *S. douthitti* are resistant to a second exposure of *S. douthitti;* however, a previous exposure of *S. douthitti* does not protect an animal from infection with

Schistosoma mansoni. From the pathology of the lungs at necropsy it appears that in resistant animals cercariae do not reach the lungs.

The susceptibility of primates for animal schistosomiasis and the problem of the susceptibility of men toward infection with animal schistosomes is discussed.

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